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(54) Title: NUCLEIC ACID AMPLIFICATION BY TWO-ENZYME, SELF-SUSTAINED SEQUENCE REPLICATION

(57) Abstract

Novel methods are provided for nucleic acid amplification by continuous, substantially isothermal, self-sustained sequence replication ("3SR") utilizing RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity, RNAse H activity and DNA-dependent RNA polymerase activity. In one of the methods, before enzymatic activities can be provided by only two enzymes, a reverse transcriptase and a DNA-dependent RNA polymerase. The methods may employ two or three enzymes to provide the necessary enzymatic activities. Thus, in certain of the methods, an exogenous source of RNAse H, such as E. coli RNAse H, is employed in combination with a reverse transcriptase and a DNA-dependent RNA polymerase. In other of the methods of the present invention, reaction media are employed in which the inherent RNAse H activity of retroviral reverse transcriptases is effective to provide high levels of amplification so that only two enzymes, reverse transcriptase and DNA-dependent RNA polymerase, are required. Novel compositions for carrying out the methods of the present invention are also provided.

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NUCLEIC ACID AMPLIFICATION BY TWO-ENZYME, SELF-SUSTAINED SEQUENCE REPLICATION

TECHNICAL FIELD

The present invention relates generally to methods and kits for amplifying target nucleic acid segments in samples of nucleic acids. The invention also concerns applications of the methods and kits in molecular biology, molecular genetics and nucleic acid probe hybridization assays, including such assays employed in diagnoses of diseases.

BACKGROUND OF THE INVENTION

Much of the work in molecular biology, molecular genetics and applications thereof, such as use of nucleic acid probe hybridization assays for diagnosing diseases by detecting blood-borne pathogens or defective genes, involves the detection or isolation of a particular nucleic acid sequence (i.e., a nucleic acid segment with a particular sequence) from a background of a very large number of different, but sometimes similar, sequences of the same or nearly the same length. A fundamental problem in such work is to detect or isolate, and if possible quantitate, a particular nucleic acid sequence of interest in such a background. The problem has been a difficult one because biological materials, such as cell cultures, tissue specimens and blood samples, which provide the mixtures of nucleic acids, in which a particular segment needs to be detected or from which a particular segment needs to be isolated, typically are comprised of a complex mixture of RNAs and DNAs, of which at most only a minuscule fraction has a segment of interest.

Two fundamentally different approaches have been taken to address the problem of detecting, or isolating after cloning, a nucleic acid segment of

interest ("target segment"), that is present at a low level in a complex mixture of nucleic acids.

In the first approach, the amount of nucleic acid (including the target segment) in a sample of nucleic acid subjected to analysis is not altered; instead, a signal-generating system is associated with the target segment and produces a detectable signal representative of the presence or the number of copies of target segment in the sample. For example, a nucleic acid probe, with a sequence complementary of that of at least a subsegment of the target segment and linked to an enzyme, such as alkaline phosphatase, is mixed with sample under hybridization conditions, that effect hybridization between the probe and target segment but not appreciably between probe and other nucleic acid segments in the sample. After removing probe that fails to hybridize, a substrate for the enzyme (e.g., a chromogenic substrate for alkaline phosphatase) is added under conditions which allow catalysis by the enzyme to proceed and, in principle, a large number of detectable molecules is rapidly produced in the enzyme-catalyzed reaction (visibly colored in the case of a chromogenic substrate with alkaline phosphatase) for each probe molecule hybridized to target segment.

Numerous other systems for detecting nucleic acid segments without altering the amount of target nucleic acid in the sample are known to the art. For example, target nucleic acid segments have been detected on the basis of hybridization with a probe labelled with a radioactive isotope (e.g., ^{32}P) or a fluorescent moiety. Alternatively, it is known to use a probe which comprises or is linked to an autocatalytically replicatable RNA molecule (e.g., an RNA that is a substrate for the RNA-dependent RNA polymerase of Qβ phage or brome mosaic virus (BMV, see Miele et al., J. Mol. Biol. 171, 281 (1983)) and, after hybridization of probe with nucleic

acid of a sample and then washing unhybridized probe from the sample, to induce replication of the replicatable RNA with the corresponding RNA polymerase and, finally, detect replicated RNA molecules. A system in which probe for a target segment is linked to a RNA capable of being replicated by Qβ replicase is described by Chu et al., Nucl. Acids Res. 14, 5591 (1986) and United States Patent No. 4,957,858 and by BMV replicase by Marsh et al., Positive Strand RNV Viruses (Proceedings of 1986 UCLA Symposium), Alan R. Liss Publ. Co., New York, New York (1987).

This first approach, of amplifying signals associated with a target, has two serious drawbacks. First, in many instances, the copy number of target segment in a sample of practical size is so low that, even for reasonably rapid signal-generating systems, the time required to generate detectable signal that is significantly above background is impracticably long. Second, in any assay for a target segment, a signal due to "background" is unavoidable. In a system where signal is amplified, signal generation and amplification occur at essentially the same rates from "background" signal-generating molecules (e.g., probe molecules hybridized to segments with sequences nearly the same but not identical to the sequence of target segment, probe molecules adhering to glass, plastic or other components of a system, etc.) as from signal-generating molecules actually associated with target. Thus, the sensitivity of assays using the first approach is fundamentally limited by unavoidable "background" signal-generating molecules.

The second approach is fundamentally different. It involves increasing the copy number of the target segment itself, preferably to an extent greater than that of other segments in a sample, particularly those that might erroneously be detected as target segments because

of similarities in sequences.

Examples of this second approach include various culture techniques in which cells that harbor the target segment are caused to increase in number, sometimes more rapidly than other cells, or in which particular nucleic acids (e.g., plasmids, RNAs), which comprise target segment, are caused to increase in number.

Another example of this second approach is amplification of a DNA target segment in a so-called "polymerase chain reaction" ("PCR"). This technique is an adaptation of long known, naturally occurring processes in the replication of, for example, genomes of certain single-stranded DNA viruses and, in all events, is akin to DNA preparation following Hong, Bioscience Reports 1, 243 (1981); Cooke et al., J. Biol. Chem. 255, 6502 (1980); and Zoller et al., Methods in Enzymology 100, 468-500 (1983). By the PCR technique, a particular segment increases in copy number exponentially with a number of cycles, each of which entails (1) annealing to the 3'-terminal subsegment of each of the target segment and its complement (i.e., the segment of sequence complementary to that of target segment) a DNA primer, (2) extending each of the primers with a DNA polymerase, and (3) rendering single-stranded by thermal denaturation the duplexes resulting from step (2). The PCR technique is described in Saiki et al., Science 230, 135 (1985) and Mullis et al., European Patent Application Publication Nos. 0 200 362 and 0 201 184 and US Patent Nos. 4,683,195 and 4,683,202.

Another technique for carrying out the second approach to detecting a target segment present at a low level in a complex mixture of nucleic acids is by employing the so-called transcription-based amplification system ("TAS"). TAS employs an RNA-transcript-production step from a DNA synthesized to incorporate a segment with

the sequence of target and a promoter positioned, with respect to the target-sequence-containing segment, to enable transcription from the segment of a RNA with the sequence complementary to that of target. Multiple cycles can be carried out, as the RNA made in the transcription step can serve as template for making similarly transcribable DNA, which, in turn, can be transcribed to yield additional RNA. Amplification proceeds very rapidly with each cycle, as between about 10 and about 1,000 copies of RNA comprising the sequence of target segment or the sequence complementary thereto are produced rapidly from each double-stranded DNA which incorporates a promoter driving transcription of a segment comprising target segment. The TAS method is described in commonly owned United States Patent Application Serial Nos. 064,141, filed June 19, 1987, and 202,978, filed June 6, 1988 (published in International Patent Application Publication No. WO88/10315), the disclosures of which are hereby incorporated by reference. The TAS method of target nucleic acid amplification provides a rapid increase in copy number of a selected target segment by making use of two properties of DNA-dependent RNA polymerases: (1) appreciable initiation of transcription from only a small number of sequences specific for each polymerase, see, e.g., Brom et al., Nucl. Acids Res. 14, 3521 (1986); and (2) rapid production of a large number of transcripts (typically 10^2-10^4 per hour) from each copy of a promoter recognized by an RNA polymerase. See Milligan et al., Nucl. Acids Res. 15, 8783 (1987). In addition, by employing a standardization technique, use of the TAS system makes possible unambiguous measurement of the amount of target nucleic acid segment present in a sample.

The TAS method utilizes RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity, both of which can be provided by a reverse

transcriptase, as well as DNA-dependent RNA polymerase activity and primers. The primers define the ends of the target segment to be amplified. At least one of the primers, typically that which hybridizes to the 3'-end of the target segment, includes a segment which has the sequence of the sense strand of a promoter and is operatively linked for transcription to the segment of the primer with the sequence complementary to that of the 3'-end of the target segment, to initiate transcription in the double-stranded DNA, which comprises the promoter and target segment. Exemplary promoters employed in the TAS method are those recognized by the RNA polymerases of T7 phage, T3 phage, and SP6 phage.

The TAS method can be employed to amplify an RNA target segment. In such amplifications, the primers are employed to make from the RNA comprising the target segment a double stranded DNA which incorporates a promoter driving transcription of a DNA which comprises a segment with the sequence of target segment, to yield RNA comprising a segment with the sequence complementary to that target segment.

The TAS method can also be employed to amplify a target segment of a double-stranded nucleic acid. Briefly, the double-stranded nucleic acid of a sample is denatured and the primers are allowed to hybridize to their respective strands, one primer (the "antisense" primer) hybridizing to the 3'-end of target segment and the other (the "sense" primer) to the 3'-end of the complement of target segment. The primers are then extended with a suitable polymerase and the resulting duplexes are thermally denatured and cooled to allow the respective primers to hybridize again, to not only the strands of double-stranded sample nucleic acid which comprise target segment but also to the extension products made in the initial primer extension reaction. The hybridized primers are again extended in a reaction

catalyzed by a suitable polymerase and, with the primers hybridized to extension products of the initial primer extension, two types of double-stranded DNA are formed, at least one of which comprises a promoter operatively linked for transcription to a segment which comprises target segment. The double-stranded DNAs, which comprise such promoters, are transcribed by a DNA-dependent RNA polymerase which recognizes the promoter, to yield RNA comprising a segment complementary to that of target segment and, thereby, in effect, to amplify target segment itself. The above process of hybridization, extension, thermal denaturation, hybridization, extension and transcription may be repeated using both the strands of the newly produced double-stranded DNAs and the resulting RNA transcripts as templates.

The TAS method of amplification, unless autocatalytic replication of RNAs made in the process is employed, yields, inter alia, a first single-stranded RNA transcript, which comprises a segment with the sequence of either target segment or the complement thereof, and which is in large excess relative to a second RNA of sequence complementary to that of the first RNA. Thus, TAS provides an abundance of single-stranded RNA which can be detected without the necessity of cumbersome, repeated PCR thermal cycling or strand separation.

It would be desirable to provide a form of transcription based amplification which eliminates the need for a thermal denaturation step during each round of amplification such that multiple rounds of amplification may proceed without thermal denaturation. Thus, it would be very desirable to provide a form of transcription based amplification which is self-sustained and proceeds isothermally.

SUMMARY OF THE INVENTION

The present invention entails the surprising

discovery of a method of substantially continuous, self-sustained, target nucleic acid amplification which proceeds spontaneously and isothermally. This method for self-sustained sequence replication (hereinafter "3SR") provides for amplification of an RNA target segment utilizing RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity, RNAse H activity and DNA-dependent RNA polymerase activity, and primers which are capable of hybridizing to the target segment or complement thereof and priming a primer extension reaction utilizing, as the template, the target segment or complement thereof. At least one of the primers provides a promoter sense sequence. The RNAse H activity obviates the need for thermal cycling by enzymatically catalyzing the digestion of an RNA strand of an RNA-DNA duplex rendering single stranded the DNA strand of said duplex which was synthesized in a primer extension reaction utilizing said RNA strand as template. The four enzymatic activities may be provided by a combination of reverse transcriptase and DNA dependent RNA polymerase. The present invention entails methods of 3SR amplification wherein the inherent RNAse H activity of reverse transciptase provides the required RNAse H activity and methods wherein the RNAse H activity of reverse transcriptase is supplemented with another source of RNAse H activity, such as E. coli RNAse H, whereby increased levels of amplification of from about 10^5- to 10^6-fold may be achieved.

The present invention also entails the surprising discovery that, under certain reaction conditions, reverse transcriptases have sufficient RNAse H activity to provide extremely sensitive 3SR amplification reactions which are capable of amplifying an RNA target segment from about 10^5-fold to about 10^9-fold in less than 4 hours without supplementing the reaction medium with a source of RNAse H activity other

than the reverse transcriptase. In the absence of said certain reaction conditions 3SR amplification levels greater than about 10^3- to 10^4-fold are not attainable unless the RNAse H activity of reverse transcriptase is supplemented with RNAse H activity from, for example, E. coli RNAse H. Thus, in another of its aspects, the present invention relates to 2-enzyme 3SR methods of target nucleic acid segment amplification which enable a target nucleic acid segment to be amplified from about 10^5-fold to about 10^9-fold within 4 hours, typically in from 1/2 hour to 2 hours.

The present invention is further concerned with novel improvements in 3SR amplification methods. These improvements entail improved reaction media and other reaction conditions, which enable target segment amplification by 3SR to proceed with only two enzymes, and provide for increased levels of amplification in both 2-enzyme 3SR and 3-enzyme 3SR reactions.

The present invention also provides methods for 2-enzyme 3SR amplification, whereby relatively large target segments, in excess of about 700 bases, may be amplified to levels otherwise achievable with only smaller target segments.

The present invention provides kits for amplification by 3SR of target nucleic acid segments and for assays of samples for the presence of target nucleic acids by methods comprising amplification by 3SR, said kits comprising improved reaction media for 3-enzyme 3SR amplification or components for 2-enzyme 3SR amplification.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of an embodiment of the present invention. Figure 1 depicts the process of self-sustained sequence replication (3SR) as a step-by-step process, although it will be understood

that in practice all of the various steps occur simultaneously in a highly interactive fashion. The enzyme activities necessary for the 3SR amplification reaction are RNA-dependent DNA polymerase activity (RT activity 1), DNA-dependent DNA polymerase activity (RT activity 2), RNAse H activity (RT activity 3), and DNA-dependent RNA polymerase activity. The darkened rectangular blocks represent promoter-providing segments of the first primer (designated "A") and the second primer (designated "B").

Figures 2a and 2b depict a detailed schematic representation of the various steps of an embodiment of the present invention which shows the various subsegments which comprise the nucleic acid species stably or transiently present during the 3SR amplification reaction, as described hereinafter.

DETAILED DESCRIPTION OF THE INVENTION

Commonly owned United States Patent Application Serial No. 285,467, filed December 16, 1988, which is incorporated, in its entirety, herein by reference, discloses a method of substantially continuous, self-sustained, target nucleic acid amplification which proceeds spontaneously and isothermally. This method advantageously avoids the need for thermal denaturation of hybridized nucleic acids. Because this amplification method proceeds spontaneously and isothermally in the presence of target RNA segment, the required primers, the enzymes providing the necessary enzymatic activities, and nucleoside triphosphate substrates, the method is named "self-sustained sequence replication," hereinafter abbreviated as "3SR".

Self-sustained sequence replication can be made to proceed to completion because, as was discovered surprisingly, it is possible to maintain a reaction mixture, including enzymes for providing the four

necessary enzymatic activities, primers, ribonucleoside triphosphates and 2'-deoxyribonucleoside triphosphates, and RNA, under reaction conditions suitable for both hybridization of primers and, at suitable levels, the four necessary enzymatic activities.

The 3SR method employs two DNA primers, which prime chain-extension reactions using the target segment or complement thereof, respectively, as template. At least one of the primers includes the sense strand of a promoter. Amplification by the 3SR method, which is continuous and substantially isothermal, requires four enzymatic activities provided by at least two enzymes - reverse transcriptase (to provide RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity, and RNAse H activity) and a DNA-dependent RNA polymerase. The RNAse H activity employed in 3SR is used to render single-stranded a DNA extension product when an RNA segment acts as template for making the extension product, unlike TAS, which requires a denaturation step. The RNAse H activity of a reverse transcriptase used in the 3SR reactions of the invention may optionally be supplemented with a source of RNAse H activity other than the reverse transcriptase, such as E. coli RNAse H. However, because E.coli RNAse H has its own optimal reaction conditions which differ from the optimal reaction conditions of the other two enzymes, and because E. coli RNAse H is not easily isolated in acceptably pure form, and is considerably more expensive than the other two enzymes required for 3SR amplification, it is highly desirable to eliminate the requirement for an enzyme separate from a reverse transcriptase to provide an amount of RNAse H activity effective for high sensitivity amplification which is necessary to detect nucleic acid target segments present, before amplification, at very low concentrations.

Reference is made to standard textbooks of

molecular biology that contain definitions and methods and means for carrying out basic techniques of the present invention such as: DNA probe or primer preparation, including DNA synthesis; hybridization methodology including variations in stringency conditions for producing more or less hybridization specificity depending upon the degree of homology of a primer to a target DNA segment; RNA- and DNA-dependent DNA polymerization reactions and synthesis of cDNAs; identification, isolation, sequencing or preparation of promoters, or more specifically, promoters or sites recognized by bacteriophage DNA-dependent RNA polymerases for binding preparatory to catalysis of transcription, or, in the employment of eukaryotic systems, such promoters or sites recognized by viral DNA- and RNA-dependent RNA polymerases, for example, adenovirus-encoded RNA polymerase and brome mosaic virus RNA polymerase; conditions conducive to the production of RNA transcripts, including so-called transcription enhancer sequences; polymerase chain reaction methods including the reagents used therein; and so forth. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, (1982), and the various references cited therein; U.S. Patent 4683195; U.S. Patent 4683202; Beaucage et al., Tetrahedron Letters 22, 1859 (1981); Caruthers et al., Meth. Enzym. 154, 287 (1985); Lee et al., Science 239, 1288 (1988); Milligan et al., Nucleic Acids Res. 15, 8783 (1987); Miller et al., Virology 125, 236 (1983), Ahlquist et al., J. Mol. Biol. 153, 23 (1981); Miller et al., Nature 313, 68 (1985); Ahlquist et al., J. Mol. Biol. 172, 369 (1984); Ahlquist et al., Plant Mol. Biol. 3, 37 (1984); Ou et al., PNAS 79, 5235 (1982); Chu et al., Nucl. Acids Res. 14, 5591 (1986); European Patent Application Publn. No. (EPA) 194809; Marsh et al., Positive Strand RNA Viruses, p. 327-336, Alan R. Liss

(publ.; New York) (1987; Proceedings of UCLA Symposium, 1986); Miller et al., J. Mol. Biol. 187, 537 (1986); Stoflet et al., Science 239, 491 (1988); and Murakawa et al., DNA 7, 287 (1988).

All of the aforecited publications are by this reference hereby incorporated by reference herein.

By the term "primer" in the present context is meant a single-stranded nucleic acid that has a segment at its 3'-end with sufficient homology to a segment of the target segment or complement thereof such that, under suitable hybridization conditions, it is capable of hybridizing to the target segment (or complement thereof) and priming a primer extension reaction in which a nucleic acid having the sequence of the target segment (or complement thereof) is the template. A hybridizing segment of a typical primer is at least about 10 nucleotides in length, more preferably 15-50 nucleotides, and most preferably approximately 15-25 nucleotide bases in length. A "primer" is preferably a DNA.

As used herein an "antisense primer" means a primer which has a sequence sufficiently complementary to a sequence at the 3'-end of the target segment to be extended in a chain-extension reaction using target segment as template; a "sense primer" means a primer which has a sequence similarly sufficiently homologous to a sequence at the 5'-end of such target segment. The primers define the ends of the target segment to be amplified. In the most preferred embodiments, the sense and antisense primers, respectively, have segments, which include at least their 3'-ends, that share identity or very high homology with the 5'-end of the target segment and the complement of the 3'-end of the target segment, respectively. See, for example, EPA 128042 (publd. 12 Dec 84).

At least one, optionally both, of the primers comprise a segment with a promoter sense sequence. By

the term "promoter sense strand" is meant a single stranded nucleic acid which, when hybridized with its complement to be in its double-stranded form (i.e., as a double-stranded promoter), is specifically recognized by an RNA polymerase, which binds to a polymerase-binding sequence of the promoter and initiates the process of transcription whereby an RNA transcript is produced. In principle, any sense promoter sequence may be employed for which there is a known and available polymerase that is capable of recognizing the sequence. Typically, known and useful promoters are those that are recognized by certain bacteriophage RNA polymerases, such as those from bacteriophage T3, T7 or SP6. See Siebenlist et al., Cell 20, 269 (1980). These are but examples of the RNA polymerases which can be employed in the practice of the present invention in conjunction with their associated promoter sequences. Also, a "promoter sense strand," as used herein, preferably comprises one or more nucleotides, more preferably about 4 to about 10 or more nucleotides, adjoining the 5'-most nucleotide of the promoter (sense-strand) consensus sequence (i.e., the sense sequence of the consensus polymerase-binding site). As used herein, a "promoter sense sequence" must be of sufficient length such that, upon completion of a cDNA incorporating said sequence, the consensus sequence of the promoter is completely double-stranded. In these cDNAs, transcription occurs from the promoter when an RNA polymerase that recognizes the promoter is present under conditions suitable for transcription from the promoter.

Bacteriophage promoters are preferred because of their high specificity for their cognate RNA polymerases. Other promoters and their corresponding DNA-dependent RNA polymerases which have similarly high specificity could be employed in accordance with the invention in place of the bacteriophage promoter polymerases, and the invention is intended to cover such

other promoters and RNA polymerases as well, provided that said promoter shows a high degree of specificity for said polymerase.

The preferred of the bacteriophage promoter sense sequences are the (+) strands of T7, T3 and SP6 promoters which include the segment to which the corresponding RNA polymerase binds and at least one, and preferably about 4 up to about 10, nucleotides 5' from the 5'-end of this polymerase-binding segment. Preferred promoters and their corresponding RNA polymerases are described in the examples and claims, but numerous other promoters and RNA polymerases are known in the art and can be employed as well.

The "variable subsegments" that are optionally included in the DNA primers serve one or more functions. First, for the primer(s) which include(s) a promoter sequence, the variable subsegments preferably include transcription initiation sequences that are preferred by the RNA polymerase corresponding to the promoter. While the bacteriophage T7 transcription initiation sequence 5'-GGGA-3', which is located adjacent to the 3' end of the 17 nucleotide T7 promoter consensus sequence, is believed to be important to <u>in vivo</u> transcription, it does not appear to be crucial for transcription during 3SR amplification. Example IX, below shows the effect on amplification levels caused by mutations (nucleotide changes or deletions) in the transcription initiation sequence immediately downstream from the 17 nt consensus sequence of the T7 promoter. The transcription initiation sequence is optional for primers having a promoter-providing segment. Such primers having the 3'-most nucleotide of the promoter consensus sequence adjoining a target hybridization segment may provide high levels of amplification comparable to those attained where the transcription initiation sequence 5'-GGGA-3' is present. It is preferred, however, to include a segment

of at least one to about four, preferably four, nucleotides adjoining the 3'-most nucleotide of the promoter consensus sequence. An example of a preferred sequence for inclusion at the site of the transcription initiation sequence adjoining the 3'-most nucleotide of the T7 consensus sequence is the sequence 5'-GAAA-3'.

Second, for all of the primers, a variable subsegment can optionally contain a particular non-target segment whereby RNA product from the amplification can be detected in a nucleic acid probe hybridization assay. Indeed, amplification (and assay) can occur for several different target segments simultaneously by using sets of primers that differ in their recognition ("anti-target" or "anti-target complement") segments, at their 3'-ends but include a common variable subsegment. The variable subsegment may also contain a polylinker sequence that conveniently contains a plurality of restriction sites for ease of subsequent cloning. Further, the variable subsegment may contain the sequence of a self-replicatable RNA, such as Qβ virus, which, in the presence of its corresponding replicase (e.g., Qβ replicase), can multiply and autoreplicate an RNA transcript having such variable subsegment.

The term "operably linked" in particular in connection with the linkage of a promoter sequence of a primer to a hybridizing (anti-target or anti-target complement) sequence of said primer, refers to the functionality of the ultimate "double-stranded nucleic acid template" or "cDNA" synthesized in the amplification methods of the present invention and incorporating the primer. cDNAs so produced are capable of producing RNA transcripts in the presence of a DNA-dependent RNA polymerase that recognizes the promoter, when the promoter sense-strand segment of a primer is "operably linked for transcription" to the primer 3'-segment, which hybridizes to target for complement of target.

The primer extension reaction to produce a DNA-RNA or DNA-DNA duplex is well known. Reverse transcriptases, particularly from retroviruses, are known to be useful for providing DNA-dependent-DNA polymerase and RNA-dependent-DNA polymerase activity.

By a "high sensitivity amplification-effective amount of RNAse H activity" is meant an amount of RNAse H activity which, in a reaction mixture containing appropriate primers (for 3SR amplification of an RNA target segment) and RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and DNA-dependent RNA polymerase (and substrates therefor), and which is incubated in a temperature range at which the latter three enzymatic activities are active, is capable of amplifying said RNA target segment at least about 10^5-fold in 2-4 hours. The term "high sensitivity amplification-effective amount of RNAse H" as used herein is meant to relate to the level of amplification necessary in a 3SR reaction to detect, using known nucleic acid hybridization assay techniques, a nucleic acid target segment which is present, prior to amplification, in a reaction medium at a level of 1 to 10,000 molecules (e.g., in a reaction volume of about 0.05 to about 1 ml). An amount of RNAse H activity which is effective for 3SR amplification reaction may be less than a "high sensitivity amplification-effective amount" of RNAse H activity, and in such cases may be sufficient to amplify, in a 3SR amplification reaction, a target nucleic acid segment which is present at a concentration such that the level of amplification needed for detection is less than about 10^2-fold to about 10^4-fold.

The RNAse H activity known to be possessed by retroviral reverse transcriptases, is known under certain conditions, to digest the RNA strand of an RNA-DNA duplex into small oligonucleotides (e.g., oligoribonucleotides of less than about 5-10 bases in length) while leaving

the DNA strand intact. However, under reaction conditions which are described in the aforementioned, herein incorporated U.S. Serial No. 285,467, the RNAse H activity inherent in reverse transcriptases is insufficient to provide high sensitivity 3SR amplification. Under reaction conditions described in Serial No. 285,467, the addition of E.coli RNAse H supplements the inherent RNAse H activity of a reverse transcriptse and allows 3SR amplification levels necessary for detection, by nucleic acid hybridization, of a nucleic acid target segment present at a concentration of about 10 attomole/ml before amplification.

The four ribonucleoside triphosphates, rATP, rUTP, rCTP and rGTP, are referred to collectively herein as "rNTPs" or "rXTPs."

The techniques for forming a detectable signal, such as via radioactive labeling or chromogenic means using an enzyme to catalyze a chromogenic reaction, are also well known and documented in the art.

In one of its aspects, the invention entails a method for 3SR amplification of a target RNA segment of a target RNA molecule which segment comprises a 5'-subsegment, which includes a 5'-terminal nucleotide and extends at least 9 nucleotides in the 3'-direction from the 5'-terminal nucleotide of the target segment, and a 3'-subsegment, which does not overlap the 5'-subsegment and which includes a 3'-terminal nucleotide and extends at least 9 nucleotides in the 5'-direction from the 3'-terminal nucleotide of the target segment (see, e.g., Figure 2a, step 1), which method comprises incubating in a reaction medium:

(a) (1) a first DNA primer which is a single stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first

subsegment of said first primer being of the same length as the 3'-subsegment of the target segment and having a sequence sufficiently complementary to that of the 3'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence of the target segment is the template, and (2) a second DNA primer, which is a single-stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said second primer being of the same length as the 5'-subsegment of the target segment and having a sequence sufficiently homologous to that of the 5'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence complementary to that of the target segment is the template, provided that at least one of said primers further comprises a promoter-providing subsegment, which comprises the sense strand of a first promoter, said sense strand being joined to the first subsegment of the primer, which comprises said promoter-providing segment, operably for transcription from said first promoter of a cDNA comprising the extension products of said two primers, and provided further that, where said first primer lacks such a promoter-providing subsegment, then the 5'-terminal nucleotide of said 5'-subsegment of said target RNA segment is the 5'-terminal nucleotide of the target RNA molecule;

(b) at least two enzymes which exhibit in said reaction medium DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, RNAse H activity and a DNA-dependent RNA polymerase, said DNA-dependent RNA polymerase in said reaction medium, being capable of catalyzing transcription from said first promoter; and

(c) nucleoside triphosphates required as

substrates for the DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and DNA-dependent RNA polymerase activities;

wherein said incubation occurs in a range of temperatures at which said enzymes in said reaction medium are active in providing said DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, RNAse H, and DNA-dependent RNA polymerase activities.

In another of its aspects, the invention entails a method for highly sensitive, highly productive, 2-enzyme 3SR amplification of a target RNA segment of a target RNA molecule which segment comprises a 5'-subsegment, which includes a 5'-terminal nucleotide and extends at least 9 nucleotides in the 3'-direction from the 5'-terminal nucleotide of the target segment, and a 3'-subsegment, which does not overlap the 5'-subsegment and which includes a 3'-terminal nucleotide and extends at least 9 nucleotides in the 5'-direction from the 3'-terminal nucleotide of the target segment (see, e.g., Figure 2a, step 1), which method comprises incubating in a reaction medium:

(a) (1) a first DNA primer which is a single stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said first primer being of the same length as the 3'-subsegment of the target segment and having a sequence sufficiently complementary to that of the 3'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence of the target segment is the template, and (2) a second DNA primer, which is a single-stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said second primer being of the same length

as the 5'-subsegment of the target segment and having a sequence sufficiently homologous to that of the 5'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence complementary to that of the target segment is the template, provided that at least one of said primers further comprises a promoter-providing subsegment, which comprises the sense strand of a first promoter, said sense strand being joined to the first subsegment of the primer, which comprises said promoter-providing segment, operably for transcription from said first promoter of a cDNA comprising the extension products of said two primers, and provided further that, where said first primer lacks such a promoter-providing subsegment, then the 5'-terminal nucleotide of said 5'-subsegment of said target RNA segment is the 5'-terminal nucleotide of the target RNA molecule;

(b) (1) a reverse transcriptase which exhibits in said reaction medium DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and a high sensitivity amplification-effective amount of RNAse H activity, and (2) a DNA-dependent RNA polymerase which, in said reaction medium, catalyzes transcription from said first promoter; and

(c) nucleoside triphosphates required as substrates for the DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and DNA-dependent RNA polymerase activities;

wherein said incubation occurs in a range of temperatures at which said enzymes in said reaction medium are active in providing said DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, RNAse H, and DNA-dependent RNA polymerase activities.

3SR amplification methods require at least one promoter-providing primer, which is a primer which

comprises a segment with the sense sequence of a promoter, which segment is linked operably for transcription to the 3'-segment of the primer, through which the primer hybridizes to the target segment or complement of target segment. The first primer and the second primer are also referred to herein as the "antisense primer" and "sense primer," respectively. The antisense primer is the preferred primer to include a promoter sequence, although as will become clear from the description of the present invention, the antisense and the sense primers may each include a promoter sequence, and in certain instances, amplification may proceed where only the sense primer includes such a promoter sequence. The latter embodiments, in which only the sense primer comprises a sense strand of a promoter, require an RNA target segment wherein the 5' terminal nucleotide of the 5'-subsegment is also the 5'-end of the entire target RNA molecule.

Certain subscripts are employed to define the various nucleic acids stably or transiently present during 3SR amplification. These subscripts have the following meanings: "1" -- a sequence associated with a first primer; "2" -- a sequence associated with a second primer; "t" -- a portion of the target segment; "c" -- a sequence complementary to a portion of the target segment; "r" -- RNA; and "d" -- DNA. For example, the segment designated hereinafter as (3'-subsegment$_{tcd}$) is a DNA (d) sequence which is complementary (c) to the 3'-subsegment (i.e., primer hybridizing segment) of the RNA target (t) segment, which hybridizing segment is designated (3'-subsegment$_{tr}$).

2-enzyme 3SR amplification methods of the present invention wherein the antisense primer includes a promoter sense sequence will now be described. The following description assumes the presence of an RNA target segment in the reaction medium. Also described

thereafter is the production of an RNA target segment from a double-stranded DNA, which includes a segment with the same sequence as the target RNA segment (substituting ribonucleotides for 2'-deoxyribunucleotides and U for T), for situations where a target RNA segment may not be present initially in a biological sample or other sample of nucleic acid to which the method of the invention is to be applied.

With reference to Figure 1, 2-enzyme 3SR amplification, in accordance with the present invention, is initiated with a target RNA segment of Formula I:

5'-(5'-subsegment$_{tr}$)-(intermediate subsegment$_{tr}$)-(3'-subsegment$_{tr}$)-3'.

I

The subsegment designated (5'-subsegment$_{tr}$) is an RNA segment of known sequence having at least 10 nucleotides, including the 5'-most nucleotide of the RNA target segment and extending in the 3' direction therefrom at least 9 nucleotides. The (3'-subsegment$_{tr}$) is an RNA segment of known sequence of at least 10 nucleotides, including the 3'-most nucleotide of the RNA target segment and extending in the 5' direction therefrom at least 9 nucleotides. The (intermediate subsegment$_{tr}$) is an RNA segment of 0 or more nucleotides which adjoins the (3'-subsegment$_{tr}$) and the (5'-subsegment$_{tr}$). If (intermediate subsegment$_{tr}$) has 0 nucleotides, then the 3'-terminus of (5'-subsegment$_{tr}$) adjoins the 5'-terminus of (3'-subsegment$_{tr}$).

The first step in the 3SR amplification process involves hybridization of the first (antisense) primer to the 3'-subsegment of the RNA target (3'-subsegment$_{tr}$) and extension thereof by the RNA-dependent DNA polymerase activity of a reverse transcriptase using the target RNA as a template to form a DNA-RNA duplex. The so-formed

DNA-RNA duplex extends at least to the 5'-terminal nucleotide of the (5'-subsegment$_{tr}$). See Figure 2a, step 3.

The first primer is a single-stranded DNA which comprises the nucleic acid segment of Formula II:

5'-(promoter$_{1d}$)-(variable subsegment$_{1d}$)-(3'-subsegment$_{tcd}$)-3'.

II

The segment designated (promoter$_{1d}$) is a single-stranded DNA segment with the sequence of the sense strand of a first promoter, preferably one recognized by a bacteriophage DNA-dependent RNA polymerase; (3'-subsegment$_{tcd}$) is a single-stranded DNA segment having the same number of nucleotides as, and a sequence which is sufficiently complementary to, (3'-subsegment$_{tr}$) to hybridize to and prime an extension reaction using the target RNA as template; (variable subsegment$_{1d}$) is a single-stranded DNA segment of 0 to 50 nucleotides adjoining the 3'-terminal nucleotide of (promoter$_{1d}$). If said (variable subsegment$_{1d}$) has 0 nucleotides, the 3'-end of the promoter sense strand, (promoter$_{1d}$), adjoins the 5'-terminus of said (3'-subsegment$_{tcd}$). (Variable subsegment$_{1d}$) may comprise, for example, a native transcription-initiation segment recognized by the RNA polymerase which recognizes the promoter; in the case of bacteriophage T7 polymerase, this native transcription-initiation segment would have the sequence 5'-GGGA-3'. A presently preferred transcription initiation segment comprises the sequence 5'-GAAA-3'.

The improved reaction media of the present invention enable the 2-enzyme 3SR amplification methods of the present invention because such reaction media enable the expression of inherent RNAse H activity of

reverse transcriptase. This RNAse H activity of reverse transcriptase degrades the RNA strand of a DNA-RNA duplex yielding a first complementary DNA strand comprising the DNA segment of Formula III.

5'-(promoter$_{1d}$)-(variable subsegment$_{1d}$)-(3'-subsegment$_{tcd}$)-(intermediate subsegment$_{tcd}$)-(5'-subsegment$_{tcd}$)-3'.

III

The segments (intermediate subsegment$_{tcd}$) and (5'-subsegment$_{tcd}$) are the DNA segments complementary to (intermediate subsegment$_{tr}$) and (5'-subsegment$_{tr}$), respectively. The segments (promoter$_{1d}$), (variable subsegment$_{1d}$) and (3'-subsegment$_{tcd}$) are defined in Formula II.

Where the RNA target molecule is not identical in length to the RNA target segment, DNA-RNA duplex may include a DNA-RNA duplex segment which extends (relative to the RNA strand) in the 5'-direction beyond the 5'-subsegment of the target and/or single stranded RNA sequence which extends in the 3'-direction from the 3'-subsegment.

A second DNA primer ("sense primer") hybridizes to the single-stranded first complementary DNA and primes a primer extension reaction on this first complementary DNA. The second DNA primer is a single-stranded DNA comprising a sequence of at least 10 nucleotides and corresponds to Formula IV (promoter-less primer) or Formula IV(a), a promoter-containing primer:

5'-(variable subsegment$_{2d}$)-(5'-subsegment$_{td}$)-3'

IV

5'-(promoter$_{2d}$)-(variable subsegment$_{2d}$)-(5'-subsegment$_{td}$)-3'

IV(a)

The segment designated (5'-subsegment$_{td}$) is a DNA with a sequence which is sufficiently homologous to the sequence of (5'-subsegment$_{tr}$) to hybridize to a first complementary DNA (at (5'-subsegment$_{tcd}$)) and prime a primer extension reaction using a first complementary DNA of Formula III as template. The segment designated (variable subsegment$_{2d}$) is a segment of 0 to 100 nucleotides which adjoins the 5'-terminus of (5'-subsegment$_{td}$). The second promoter sense sequence is designated (promoter$_{2d}$). As with the segment designated (variable subsegment$_{1d}$), (variable subsegment$_{2d}$) may consist of a The second promoter sense sequence, if present, may be the same or different from the first promoter sequence. Where the first promoter and second promoter are different (i.e., where the first and second promoters are not recognized by the same DNA-dependent RNA polymerase) the reaction medium may optionally include a second DNA-dependent RNA polymerase which recognizes the second promoter.

Inasmuch as the segment designated (variable subsegment$_{2d}$) is an optional segment of a promoter-less sense primer of Formula IV (although it will be understood that inclusion of such subsegment may be desirable for example to provide a non-target sequence for hybridization assay purposes), this optional segment will be omitted from the description which follows which pertains to embodiments of the present invention employing a promoter-less sense primer.

The DNA-dependent DNA polymerase activity of reverse transcriptase extends the second primer using the first complementary DNA as a template to form a first double stranded cDNA (hereinafter cDNA I) which comprises

a promoter operatively linked for transcription to a cDNA segment that is the complement of the RNA target segment (i.e., has the sequence exactly complementary to that of the target segment). See Figure 2a, step 6. The cDNA comprises the first complementary DNA strand, as defined above in Formula III, and second complementary DNA strand comprising the DNA of Formula V or V(a):

5'-(5'-subsegment$_{td}$)-(intermediate subsegment$_{td}$)-(3'-subsegment$_{td}$)-(variable subsegment$_{1cd}$)-(promoter$_{1cd}$)-3',

V

5'-(promoter$_{2d}$)-(variable subsegment$_{2d}$)-(5'-subsegment$_{td}$) -(intermediate subsegment$_{td}$)-(3'-subsegment$_{td}$)-(variable subsegment$_{1cd}$)-(promoter$_{1cd}$)-3'.

V(a)

The segments (intermediate subsegment$_{td}$), (3'-subsegment$_{td}$), (variable subsegment$_{1cd}$) and (promoter$_{1cd}$) are segments which are complementary to (intermediate subsegment$_{tcd}$), (3'-subsegment$_{tcd}$), (variable subsegment$_{1d}$) and (promoter$_{1d}$), respectively. The segments (promoter$_{2d}$), (variable subsegment$_{2d}$) and (5'-subsegment$_{td}$) are defined as in Formulae IV and IV(a);

cDNA I consisting of the first and second complementary DNA strands is transcribed from the first promoter in the presence of corresponding DNA-dependent RNA polymerase to produce multiple copies of an RNA transcript of Formula VI or Formula VI(a) (See Figure 2b, step 7):

5'-(variable subsegment$_{1r}$)-(3'-subsegment$_{tcr}$)-(intermediate subsegment$_{tcr}$)-(5'-subsegment$_{tcr}$)-3'

VI

5'-(variable subsegment$_{1r}$)- (3'-subsegment$_{tcr}$)-
(intermediate subsegment$_{tcr}$)-(5'-subsegment$_{tcr}$)-
(variable subsegment$_{2cr}$)-(promoter$_{2cr}$)-3'.

VI(a)

The segment (variable subsegment$_{1r}$) is an RNA segment corresponding to (variable subsegment$_{1d}$); the segments (3'-subsegment$_{tcr}$), (intermediate subsegment$_{tcr}$), (5'-subsegment$_{tcr}$), (variable subsegment$_{2cr}$), and (promoter$_{2cr}$) are complementary to (3'-subsegment$_{tr}$), (intermediate subsegment$_{tr}$), (5'-subsegment$_{tr}$), (variable subsegment$_{2d}$) and (promoter$_{2d}$), respectively.

Each of the multiple copies of the RNA transcript of Formula VI or VI(a) is capable of hybridizing with second primer of Formula IV or IV(a), respectively, and functioning as template for a primer extension reaction to form a DNA-RNA duplex. See Figure 2b, steps 8 and 9. The inherent RNAse H activity of reverse transcriptase digests the RNA transcript strand of the DNA-RNA duplex, thus rendering single stranded the third complementary DNA of Formula VII or Formula VII(a):

5'-
(5'-subsegment$_{td}$)-(intermediate subsegment$_{td}$)-
(3'-subsegment$_{td}$)-(variable subsegment$_{1cd}$)-3'

VII

5'-(promoter$_{2d}$)-(variable subsegment$_{2d}$)-
(5'-subsegment$_{td}$)-(intermediate subsegment$_{td}$)-
(3'-subsegment$_{td}$)-(variable subsegment$_{1cd}$)-3'.

VII(a)

Each of the segments is defined as in Formulae V or V(a)

above.

Next, a first primer of Formula II hybridizes with this third complementary DNA strand and is extended to form a fourth complementary DNA. See Figure 2b, step 12. The fourth complementary DNA is a segment of Formula VIII:

$3'-(5'-\text{subsegment}_{tcd})-(\text{intermediate subsegment}_{tcd})-(3'-\text{subsegment}_{tcd})-(\text{variable subsegment}_{1d})-(\text{promoter}_{1d})-5'$

VIII

or of Formula VIII(a)

$3'-(\text{promoter}_{2cd})-(\text{variable subsegment}_{2cd})-(5'-\text{subsegment}_{tcd})-(\text{intermediate subsegment}_{tcd})-(3'-\text{subsegment}_{tcd})-(\text{variable subsegment}_{1d})-(\text{promoter}_{1d})-5'$

VIII(a)

Each of the segments is defined in Formula III above. The overhanging end of the fourth complementary DNA, the promoter-encoding sequence, acts as a template for extension of the third complementary DNA to complete a cDNA II, Formula X, which consists of a third complementary DNA strand of Formula VII and a fourth complementary strand of Formula VIII (or Formulae VII(a) and VIII(a)).

In the just-described embodiment of the present invention transcription produces either antisense transcripts (where only the antisense primer contains a promoter sense strand) or both sense and antisense transcripts (where each primer contains a promoter sense strand).

Sense transcripts are of Formula IX:

5'-(variable subsegment$_{2r}$)-
(5'-subsegment$_{tr}$)-(intermediate subsegment$_{tr}$)-
(3'-subsegment$_{tr}$)-(variable subsegment$_{1cr}$)- (promoter$_{1cr}$)-3'

IX

The segments designated (5'-subsegment$_{tr}$), (intermediate subsegment$_{tr}$) and (3'-subsegment$_{tr}$) are identical to or substantially homologous to the RNA target segment of Formula I. The segments (variable subsegment$_{1cr}$) and (promoter$_{1cr}$), respectively, are the RNA sequences complementary to (variable subsegment$_{1d}$) and (promoter$_{1d}$).

The antisense transcripts reenter the antisense amplification loop as template to produce additional copies of cDNA II. Figure 2b, steps 7-12.

The sense transcripts of Formula IX enter a discrete sense amplification loop analogous to the just-described antisense loop. Briefly, each of the multiple copies of the sense transcript is capable of hybridizing with first primer of Formula II and functioning as template for an extension product to form a DNA-RNA duplex comprising a fifth complementary DNA which is rendered single-stranded by inherent RNAse H activity of reverse transcriptase. See Figure 2b, steps 7a-10a. A second primer of Formula IV(a) hybridizes therewith and is extended to form a double stranded cDNA II which is identical to the cDNA II which consists of DNA strands of Formulae VII(a) and VIII(a). cDNA II has a completely double stranded promoter at each of its ends. (Figure 2b, steps 11a-12). Transcription may proceed from each of the two DNA strands, to produce multiple copies of sense transcripts (i.e., transcripts comprising a segment with sequence of target segments) and antisense transcripts (i.e., transcripts comprising a segment with the sequence complementary to that of target segment) to feed the two complementary amplification

loops.

Through the above-described reaction cycle one or more molecules of RNA containing a target segment may be amplified within 2 hours to 10^6 copies or more of an RNA transcript having a segment with the sequence of the target segment or the complement thereof without the need for thermal cycling or the repeated addition of enzymes. The 3SR reaction of the present invention, carried out in the improved reaction media of the present invention, requires only two enzymes, reverse transcriptase and DNA-dependent RNA polymerase, to provide the necessary four enzyme activities.

Although the foregoing description of the process was step-by-step, it will be understood that in practice all of the various steps occur simultaneously in a very complex, highly interactive fashion.

While the above described amplification mechanism employs an antisense primer containing a promoter-providing segment (i.e., includes a promoter sense sequence), 3SR amplification may also be carried out where the sense primer comprises a promoter-providing segment and the antisense primer does not. In this embodiment the RNA target molecule should not extend in the 5'-direction beyond the 5'-nucleotide of the RNA target segment (i.e., the 5'-nucleotide of the subsegment designated (5'-subsegment$_{tr}$) should be the 5'-nucleotide of the target molecule). Where the 5'-terminal nucleotide of the target segment is the 5'-terminal nucleotide of the entire target RNA molecule, amplification will proceed by steps which are analogous to those depicted in Figures 2a and 2b, but the transcripts produced will be sense transcripts which will directly enter the amplification loop depicted by steps 7a-12. No antisense transcripts will be produced.

One way to ensure that the 5'-terminal nucleotide of the RNA target molecule is the 5'-terminal

nucleotide of the RNA target segment is to amplify an RNA target segment of predetermined nucleic acid sequence which segment is at the 5'-end of the RNA target molecule. In this way a suitable sequence for the promoter-providing sense primer may be provided: For example, a suitable sense primer may comprise a DNA segment which includes a promoter sense strand operatively linked for transcription to a segment having a sequence homologous to e.g., the 20 nucleotide segment at the 5'-end of the RNA target molecule including the 5'-terminal nucleotide of the RNA target molecule and extending 19 nucleotides in the 3'-direction therefrom. The antisense primer may consist of a DNA segment having a sequence which is complementary to a segment at the 3'-end of the target segment.

A second way to provide an RNA segment meeting the limitation that the 5'-terminal nucleotide of the target RNA molecule is the 5'-terminal nucleotide of the target segment is to produce such an RNA target molecule, from a DNA segment which encodes the target sequence, by conducting a cycle of TAS amplification. An appropriate target RNA segment thus may be generated from a double stranded DNA (or single stranded DNA or RNA) known to encode the target sequence. For example, where a double stranded DNA encodes the target segment of interest, the first and second DNA primers are added to the reaction solution and the solution is heated at about 94°C - 100°C for 1 minute and is then cooled to 42°C over the course of 1 minute. This heating and then holding at 42°C, in combination with the composition of the solution, provides conditions of stringency sufficient to provide hybridization of the first primer and the second primer to the two complementary strands of said double-stranded DNA, with sufficient stability to prime a primer extension reaction. Reverse transcriptase or DNA-dependent DNA polymerase (and the necessary

nucleoside triphosphates, if not previously added) is added to polymerize the extension reaction. The heat-stable DNA polymerase from Thermus aquaticus (see Chien et al. J. Bacteriol. 127, 1550 (1976)), Sequenase™ brand recombinant T7 DNA polymerase from the U. S. Biochemicals Corp., Cleveland, Ohio, U.S.A., and the well known Klenow Fragment of E. coli DNA polymerase I, and calf thymus DNA polymerase alpha, may be used.

The nucleic acid containing solution is again heated to 100°C for 1 minute and cooled to 42° for 1 minute during which step first and second primers hybridize with the extension products of the prior primer extension reaction. In the case of 2-enzyme 3SR, reverse transcriptase and DNA-dependent RNA polymerase are then added such that the hybridized primers are extended to complete the cDNA synthesis and the cDNA is transcribed to produce target RNA transcripts. 3SR amplification is thereby initiated.

The target RNA segment provided by such a cycle of TAS amplification has its 5'-end and 3'-end defined by the two primers utilized. Such an RNA therefore satisfies the requirement that the 5'-terminal nucleotide of the RNA target molecule is the 5'-terminal nucleotide of the 5'-subsegment of the target RNA. It should also be clear, however, that such a cycle of TAS amplification may virtually always be used to produce an RNA target segment suitable for 3SR amplification, without regard to which primer(s) include(s) a promoter sequence. See Example II.

In preferred embodiments of the present invention, the amplifiable target segment of a nucleic acid of interest has an (intermediate subsegment$_{tr}$) including at least 20, and more preferably at least about 50, nucleotides to permit, optionally, the use of a second round of 3SR amplification using third and fourth DNA primers to amplify the (intermediate subsegment$_{tr}$)) in

a further refinement of the amplification method. Also, the (intermediate subsegment$_{tr}$) may be used for detecting 3SR-produced transcripts by a nucleic acid hybridization assay, where the (variable subsegment) does not have a non-target segment which may be used for this purpose.

In several of its aspects, the present invention involves the discovery that two enzymes, a retroviral reverse transcriptase and a DNA-dependent RNA polymerase by themselves and in the absence of an exogenous any source of RNAse H activity other than the reverse transcriptase, can provide the four enzyme activities necessary for high sensitivity 3SR amplification, which requires a high sensitivity amplification effective amount of RNAse H activity. One such method for augmenting the endogenous RNAse H activity of the reverse transcriptase entails carrying out the reaction in a reaction medium comprising about 20 to 40 mM of a magnesium-containing salt such as magnesium chloride, magnesium sulfate and the like; about 1 to 25 mM of an alkali metal chloride such as KCl or NaCl and the like; about 0 to 20 mM of a sulfhydryl reducing agent such as dithiothreitol (DTT), beta mercaptoethanol and the like; about 0 to 10 mM spermidine; about 1 to 8 mM ribonucleoside triphosphates; about 1 μM to 8 mM 2'-deoxyribonucleoside triphosphates and about 0 to 25 volume percent of a sulfoxide compound such as dimethylsulfoxide.

Preferred reaction media comprise:

20 - 40 mM $MgCl_2$
1 - 25 mM KCl
1 - 10 mM Spermidine
1 - 20 mM DTT
1 - 7 mM rNTPs
0.01 - 2 mM dNTPs
0 - 15% dimethylsulfoxide (by volume)

and an appropriate buffer (Tris, HEPES, etc.) such that said reaction medium has a pH of between about 7.5 and about 8.5, preferably pH 8.1. The DMSO is required when AMV reverse transcriptase is the source of RNAse H activity.

Prior to the present invention, 3SR reactions were believed to be unsupportable using as a source of the four required enzymatic activities only a retroviral reverse transcriptase, such as AMV reverse transcriptase, and DNA-dependent RNA polymerase, where amplification levels greater than about 10^3 were desired. It has been found by the inventors that improved reaction media, surprisingly, allows the inherent RNAse H activity of retroviral reverse transcriptases to function to support such levels of 3SR amplification even in the absence of other sources of RNase H activity, such as E. coli RNAse H.

It has also been discovered by the inventors that supplementing such reaction media with from about 1 to about 25 percent by weight of a hydroxyl containing compound surprisingly increases the level of amplification which is attainable. Hydroxyl containing compounds include, but are not limited to C_1-C_{10} alcohols such as methanol, ethanol, propanol, isopropanol, butanol, isobutanol, and the like; glycols such as ethylene glycol, diethylene glycol, triethylene glycol and polyethylene glycols having an average molecular weight of up to about 20,000 daltons which are aqueous soluble; mono-, di- and trisaccharides such as glucose, galactose, mannose, fructose, sucrose, maltose, raffinose and the like; and sugar alcohols such as sorbitol, glycerol, glucitol, mannitol, inositol and the like.

The improved reaction media of the present invention therefore preferably include one or more of the following compounds: (i) a C1-C10 alcohol; (ii) a compound of the formula $HOCH_2(CHOH)_xCH_2OH$, wherein x is

0-20; (iii) a polyethylene glycol compound of the formula $H(OCH_2-CH_2)_nOH$, wherein n is 2-600, or a mixture of such compounds with an average molecular weight of about 1,000 to about 20,000, preferably about 6,000 to about 10,000, and (iv) a sugar from the group of mono-, di- and tri-saccharides and derivatives thereof. Preferred compounds of the above-defined formulae include ethanol, glycerol, sorbitol, sucrose and PEG-8000. Several of the examples below show the effect of sulfoxide and hydroxyl containing compounds on the levels of amplification attainable with 2-enzyme 3SR or 3-enzyme 3SR.

Most preferred of the reverse transcriptases (RTs) are AMV reverse transcriptase, recombinant MMLV reverse transcriptase and HIV-1 reverse transcriptase, which lack 5'-to-3' exonuclease activity.

The improved reaction media should be supplemented with a sulfoxide compound of the formula $R_1-(SO)-R_2$, wherein R_1 and R_2 are independently C_1-C_4 alkyl and wherein R_1 and R_2 can be joined as part of a saturated cyclic moiety (preferably 10% dimethylsulfoxide (DMSO) by volume) where AMV reverse transcriptase is employed for 2-enzyme 3SR, but such a sulfoxide compound may be omitted where a reverse transcriptase derived from MMLV, HIV-1, or other retrovirus is utilized.

The improved reaction media further improves amplification levels in 3SR reactions carried out in the presence of E. coli RNAse H. Again, DMSO or other sulfoxide containing compound may be used to increase amplification levels in such 3-enzyme 3SR reactions utilizing AMV reverse transcriptase.

The reaction media should be supplemented with 0.1 - 10 mM $MnCl_2$ or similar manganese salt where MMLV reverse transcriptase is employed in the 2-enzyme 3SR reactions of the present invention.

For 2-enzyme 3SR the concentration of rNTPs in the improved reaction media of the present invention

should be most preferably about 6mM although lesser concentrations of rNTPs in the improved reaction media may be employed for 3-enzyme 3SR. Unexpected increases in amplification levels of up to 10-fold or more are obtained in 2-enzyme 3SR reactions where rNTP concentration is increased from 4mM to 6mM in the improved reaction media, even though the lower concentration of rNTPs represents a large molar excess of substrate. Concentrations of rNTPs greater than about 8mM or 9mM tend to reduce the levels of amplification which may be obtained in two-enzyme 3SR reactions.

Example III, below, demonstrates the capacity of an improved reaction medium of the invention, but not a prior art reaction medium reported to support 3-enzyme 3SR, to sustain 2-enzyme 3SR.

Presently, it is preferred to use approximately 10 units of AMV reverse transcriptase and 20 units of T7 RNA polymerase per amplification reaction (100 µl) in 2-enzyme 3SR reactions. Interestingly, 3-enzyme 3SR amplification requires not only E. coli RNAse H, but also significantly higher concentrations of reverse trancsriptase (30 units) and RNA polymerase (60-100 units).

With respect to the length of a target segment, inter-primer distances of less than 1500 nucleotides are favored, presumably due to the lack of stringency under which the 3SR reactions are carried out. Generally, target segments longer than about 200 nucleotides in length may be more effectively amplified where the reaction medium is supplemented with about 1 to about 25 weight percent of an alcohol or polyhydroxy compound using either the 2-enzyme or 3-enzyme methods. It is expecially preferred to supplement the improved reaction media of the present invention with between about 5 and about 15 weight percent of sugar alcohol of the formula $HOCH_2(CHOH)_xCH_2OH$, wherein x is 0-20, more preferably,

wherein x is 0-5, and most preferably where the compound is sorbitol or glycerol.

A preferred reaction medium of the present invention for carrying out 2-enzyme 3SR amplification is an aqueous solution comprising:

BUFFER: Tris pH 8.1, 40 mM.
$MgCl_2$, 30mM,
KCl, 20 mM,
DTT, 10mM
Spermidine, 4mM.

NUCLEOTIDES: rNTPs, 6mM,
dNTPs, 1mM.

PRIMERS:

0.25 μg sense primer comprising a 15-base target binding region; and

0.25 μg antisense primer comprising a 15-base target binding region operatively linked to a promoter sequence (of about 20 bases).

ENZYMES: AMV Reverse Transcriptase, 10 units/100μl reaction solution
(reaction solution comprising 10% dimethyl sulfoxide (DMSO) and 15% sorbitol)

or

MMLV Reverse Transcriptase,
1000 Units/100 μl reaction solution
(reaction solution comprising 1 mM $MnCl_2$ and 15% sorbitol)

and

T7 RNA Polymerase, 20 units/100μl reaction solution.

Temperature of the reaction mixture during 2-enzyme or 3-enzyme 3SR amplification also has a marked effect on the level of amplification achieved. While amplification may be carried out at temperatures between about 5°C and about 50°C, more preferably amplification is carried out at between about 37°C and about 47°C and most preferably at about 42°C. Reaction temperature is particularly important in the 2-enzyme 3SR methods of the present invention, with amplification at 42°C being approximately 100-fold more effective than at 37°C. Also, amplification rates are 2- to 3-fold greater in the 42°C - 45°C temperature range in the presence of an alcohol or polyhydroxyl-containing additive such as sorbitol, glycerol, ethanol and the like, and additionally DMSO, or like sulfoxide compound where AMV reverse transcriptase is utilized.

The 3SR reaction may be more efficient in one amplification loop than in the other. Therefore, where both the sense and the antisense primers include a promoter encoding segment, either the sense or the antisense product may predominate, presumably because sequences downstream from the double stranded promoter segment of the cDNA may have a significant effect on transcription rates.

In another of its aspects the present invention concerns DNA primers capable of priming a chain elongation reaction which primers comprise a promoter sense strand having at least one to ten nucleotides extending 5' from and adjoining the 5'-most nucleotide of the segment with the sense strand of the promoter's polymerase binding site (preferably with the sequence of

the promoter's consensus sequence). The inventors have surprisingly discovered that the length and sequence of the promoter-providing segment of a primer having a promoter sense strand has a marked affect on the level of amplification in 3SR. It has been found that primers truncated at their 5'-end with the 5'-nucleotide of the promoter consensus sequence (i.e., the 5'-end of the primer is the 5'-most nucleotide of the consensus sequence) exhibit less than about 10^5-fold amplification in 1 hour at 42°C in the improved reaction media of the present invention. As understood in the art, a promoter has a number of parts. First, it has a polymerase binding segment, which is the segment of double-stranded DNA to which the polymerase binds in initiating transcription. A promoter must have at least a polymerase binding segment to function in transcription. It is thought that the consensus sequence is the minimum sequence necessary, in completely double-stranded form, which is necessary for the binding of RNA polymerase in the process of initiating transcription. Optionally, it may be desirable to include a segment referred to herein as the transcription initiation sequence immediately adjacent (downstream) to the consensus sequence. The consensus sequence for the T7 promoter is disclosed herein. Other promoter consensus sequences are well-known in the art. For example, the sense strand of the T3 consensus sequence is 5'-ATTAACCCTCACTAAA-3' and the T3 transcription initiation sequence is 5'-GGGA-3'. Also, two versions of the SP6 promoter are well-known. The consensus sequence of the sense strand of the SP6 promoter (version 1) is 5'-ATTTAGGTGACACTATA-3' and the consensus sequence of the sense strand of the SP6 promoter (version 2) is 5'-AATTAGGGGACACTATA-3'; the transcription initiation sequence for both version 1 and version 2 of the SP6 promoter is 5'-GAAG-3'. Where the initial concentration of target segment is in the

concentration range of about 0.01 - 1 attomole in a 100μl aliquot - a concentration which is not unusual for detection of the presence of, for example, HIV-1 virus or a defective gene characteristic of a disease state - such a level of amplification is not detectable by standard nucleic acid hybridization assays. However, promoter-providing primers which have as few as 1 additional nucleotide adjoining the 5'-end of the promoter consensus sequence surprisingly show about a 10-fold increase in amplification; and for each additional nucleotide up to a 4-nucleotide sequence added to the 5'-end of the consensus sequence an additional 7- to 10- fold increase is achieved. Extending an oligonucleotide from its 5'-end more than 4 nucleotides, and up to about 10 nucleotides, relative to a promoter consensus sequence may improve amplification over the level achieved with 1 to 4 nucleotides, as shown in the following Table.

The following Table demonstrates the unexpected results relating to 3SR amplification levels obtained after modifying the length and nucleotide sequence upstream from (5'-from) the promoter sense sequence of a DNA primer.

EFFECT ON 3SR AMPLIFICATIONS OF NUCLEOTIDE SEQUENCE AT 5'-END OF OLIGONUCLEOTIDE PRIMERS CONTAINING T7 PROMOTER SEQUENCES

Oligo-Nucleotide	SEQ ID No:	Length (nt)	Sequence[1]	Fold Amp.[2]
			5' -17 +1 3'	
90-425	1	59	5'AGTAATTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$3.1x10^8$
88-347	2	56	5' AATTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.9x10^8$
90-578	3	56	GATTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$6.5x10^7$
90-575	4	56	GCTTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.8x10^7$
90-577	5	56	GCGTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$5.5x10^7$
90-574	6	56	GCGCTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.1X10^8$
90-427	7	55	ATTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.2x10^8$
90-428	8	54	TTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$5.5x10^7$
90-576	9	54	GTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.5x10^7$
90-579	10	54	GCTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.1x10^7$
90-429	11	53	TTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.1x10^7$
90-205	12	52	TAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$<10^5$
90-206	13	51	AATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$<10^5$

1/ Coding strand sequence is displayed. The underlined sequence corresponds to the cannonical 17 nt T7-promoter sequence and the initiation of RNA transcription is denoted by +1. The sequence GGGA is from the T7 sequence; the +5 nucleotides onward is the HIV-1 sequence; subsequent sequences are identical for each oligonucleotide. Oligonucleotides are aligned to display differences among the primers.

2/ 3SR reactions were performed in 50µl containing 0.05 attomoles (~1,000 molecules) of HIV-1 RNA target, 30U T7 RNA polymerase, 2u *E. coli* RNAseH, 15U AMV RT, 40mM Tris, pH 8.1, 30mM MgCl2, 20mM KCl, 10mM DTT, 4mM spermidine, 1mM dXTP, 7mM rXTPs, and 0.125µg of oligononcleotide primers for 1-2 hours at 42°C. The same companion primer, 89-255 (SEQ ID No: 14) (5'TTATTGTGCCCCGGCTGGTTTTGCGATTCTA3') (Ratner et al. 1985) was used for each primer listed in this table. The RNA product made is 214 nucleotides long. Amplified targets were quantitated using beadbased sandwich hybridization (BBSH) employing 25mg of beads containing capture oligonucleotide 86-273 and 100 fmoles of ^{32}P-labeled detection of oligonucleotides 87-81 (Guatelli et al. 1990). All amplification values, except for 88-347, are the average of two 3SR reactions and each of these amplification reaction was analyzed by duplicate BBSH reactions. The 3SR amplifications performed with 88-347 represent the average of six 3SR reactions.

Amplification carried out under the above described conditions showed that, where the T7 consensus sequence was not flanked on the 5'-end by at least one nucleotide adjoining the 5'-most nucleotide of the consensus sequence, amplication was not detected at a level greater than 10^5-fold. The promoter-containing primers of the present invention having 1-10 nucleotides, and preferably 1-4 nucleotides, adjoining the 5'-terminal nucleotide of the consensus sequence significantly enhance amplification levels relative to promoter-providing primers described in the prior art.

While not intending to be bound by theory, it is believed that the one to ten nucleotide sequence adjoining the 5'-end of the consensus sequence ensures that the reverse transcriptase remains associated with the DNA template strand at least until a cDNA having a completely double stranded promoter segment is completed.

The complete double-strandedness of the promoter segment comprising the consensus sequence (presumably the polymerase-binding segment) is presumably important for efficient transcription from the promoter.

The oligonucleotide primers used in the Examples which follow, unless otherwise noted, have the nucleotide sequence corresponding to the indicated region of the HIV-1 genome as disclosed in Ratner et al., Nature (London) 313, 277-284 (1985), which is incorporated herein by reference. Unless otherwise indicated, an asterisk (*) denotes a promoter-providing oligonucleotide primer comprising the segment 5'-AATTTAATAC GACTCACTAT AGGGA-3' (SEQ ID NO: 15), wherein the underlined sequence is the 17-nucleotide consensus sequence of the promoter recognized by the T7 bacteriophage DNA-dependent RNA polymerase. The 4 nt sequence (5'-AATT-3') at the 5'-end of the consensus sequence is defined herein to be included within the term "promoter sense strand" and the 4 nt segment (e.g., 5'-GGGA-3' or 5'-GAAA-3', etc.) at

the 3'-end of the consensus sequence is the T7 transcription initiation segment, which, in the parlance adopted above, is a "variable subsegment" of the primer. Sequence corresponding to this variable subsegment will occur in transcripts made from the promoter corresponding to the promoter sense strand. For example, the promoter-containing oligonucleotide probe designated 88-347* consists of a segment which is the complement of the segment of the HIV-1 genome corresponding to nucleotides, 6661-6631 (inclusive) the 4 nt variable subsegment corresponding to the transcription initiation sequence 5'-GGGA-3', and the 21 nt promoter sense strand with the sequence given above, and has the following complete sequence:

5'-AATTTAATAC GACTCACTAT AGGGATGTAC TATTATGGTT TTAGCATTGT CTGTGA-3'. (SEQ ID NO: 2)

The nucleotide sequences of the following oligonucleotide primers (designated by primer #) are disclosed in accordance with the above convention. Several of these oligonucleotides are referred to in the specification and examples herein. A designation of "sense" means that the primer comprises a segment with the same sequence as the indicated segment from the HIV-1 genome. A designation of "Antisense" means that the primer comprises a segment that is complementary in sequence to the indicated segment from the HIV-1 genome.

Oligonucleotide Primer #	SENSE OR ANTISENSE	ENV REGION OF HIV-1 Nucleotide Positions
88-211* 1/	(sense)	6450 - 6479
89-255 (SEQ ID NO: 14)	(sense)	6450 - 6479
88-299	(sense)	6486 - 6515
89-332	(sense)	6494 - 6508
88-33	(sense)	6419 - 6440
90-106*	(sense)	6419 - 6446
88-348*	(sense)	6419 - 6446
88-347* (SEQ ID NO: 2)	(antisense)	6661 - 6631
89-263*	(antisense)	6830 - 6801
86-274	(antisense)	6691 - 6661
88-346	(antisense)	6830 - 6801
90-66	(antisense)	6918 - 6891
90-69	(antisense)	7101 - 7070
85-237	(antisense)	7255 - 7226
85-235	(antisense)	7335 - 7306
90-72*	(antisense)	7255 - 7226
90-71*	(antisense)	7335 - 7306
90-187*	(antisense)	7899 - 7870
86-273	(sense)	6591 - 6620
87-81	(sense)	6551 - 6577
87-79	(sense)	6419 - 6443

1/ In Primer# 88-211, the variable subsegment corresponding to the transcription initiation site has the sequence is 5'-GGGATC-3', instead of 5'-GGGA-3'.

In another of its aspects the invention concerns methods useful for detection of at least one specific RNA target segment in a sample containing nucleic acid, comprising amplifying said RNA target segment according to the above-recited methods and detecting the presence

of RNA transcripts which comprise a sequence that is the same as or complementary to that of said target segment. Detecting amplified nucleic acid products may be accomplished by well known nucleic acid hybridization techniques. Example II describes the bead-based sandwich hybridization technique, which is the preferred method for detecting amplification products. Among other detection methods are performing amplification using ribonucleoside triphosphates which have been labelled with a radioisotope, or a chromogenic or fluorescent substrate, or a group such as biotinyl, capable of being bound by a complex comprising an enzyme capable of catalyzing a chromogenic reaction, as well known in the art, and detecting the presence in a hybridization assay of RNA transcripts which have incorporated such labelled rNTPS.

As noted above, the invention also entails kits for carrying out the amplification methods of the invention. A kit of the invention may comprise one sense and one antisense primer (one or both including a promoter sense strand), components of a reaction medium enabling 2-enzyme 3SR amplification, and only the bacteriophage DNA-dependent RNA polymerase(s) corresponding to the promoters of the primers, and a reverse transcriptase. Alternatively, a kit of the invention may include components for 3-enzyme 3SR, including an enzyme that provides RNAse H activity but is not a reverse transcriptase, and an aqueous solution to provide an improved reaction medium of the invention or compound (e.g., salts, buffers, hydroxy compounds, DMSO, nucleoside triphosphates) to prepare such an improved reaction medium.

The invention also encompasses the improved reaction medium.

Methods and kits for carrying out nucleic acid hybridization probe assays (by amplifying a target

segment in accordance with the invention) may entail, in addition, steps and components necessary for detecting the RNA product resulting from amplification according to the invention. The skilled understand the various additional steps and components, respectively, that are required to detect RNA from an amplification process by any of the numerous nucleic acid probe hybridization assay methods known in the art. A preferred nucleic acid probe hybridization assay method, involving bead-capture of labeled amplified RNA, is illustrated in Example II below.

The invention will now be described in greater detail by way of Examples.

EXAMPLE I

PREPARATION OF TRISACRYL BEAD-BOUND OLIGONUCLEOTIDE PROBES

A 5'-aminohexyl phosphoramidate oligonucleotide derivative was prepared by reacting 5'-phosphorylated 88-297 (5'-TGGCCTAATTCCATGTGTACATTGTACTGT-3') (SEQ ID NO: 16) with 1,6 diaminohexane in the presence of 0.25 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 0.1 M methylimidazole, pH 6.0, as previously described by Chu et al., Nucleic Acids Res. 11, 6513-6529 (1983). It is essential to carry out this reaction in freshly silanized Eppendorf tubes to prevent nonspecific adsorption of nucleic acids to the walls of the tubes. The amine derivative was isolated by precipitating twice with EtOH/LiCl. Typically, a 150-µl aliquot of a 10-mg/ml solution of N-succinimidyl bromoacetate (see Bernatowicz, et al. Anal. Biochem. 155, 95-102 (1986)) in N,N-dimethylformamide was added to 2.5 nmol of 5'-aminohexyl phosphoramidate oligonucleotide derivative in 1.1 ml of 0.2 M HEPES, pH 7.7. After a reaction time of 1 h, the oligonucleotide was precipitated twice with ethanol/LiCl.

Derivatization of Trisacryl GF2000 (Réactifs IBF, Pointet Girard, France) with amino groups was performed using a 20-ml suspension of the resin, which was pipetted into a sintered glass funnel, washed with 200 ml H_2O, and sucked dry for 10 min. The dried sample (~11 g) was added slowly to 20 ml of distilled ethylenediamine that had previously been heated to 90°C in an oil bath. After 1 h at 90°C, the reaction mixture was cooled by the addition of 30 ml of crushed ice. Excess ethylenediamine was removed by successive washes of the resin in a funnel with 400 ml each of 0.2 M NaCl, 0.001 M HCl, and finally with 500 ml 0.1 M NaCl. The washes were continued until the filtrate gave a negative test with 2,4,6-trinitrobenzene sulfonic acid reagent.

Conversion of the Trisacryl-amine supports to Trisacryl-sulphydryl was carried out by first equilibrating the beads (10 g wet weight) with 0.5 M $NaHCO_3$, pH 9.7. The volume was adjusted to 40 ml in a 50-ml Sarstedt conical tube, solid N-acetyl homocysteine thiolactone (2.5 g) was added, and the tube was agitated at room temperature for 1 h. Subsequently, another gram of reagent was added, and the sample was shaken overnight. The beads were washed with 250 ml 0.1 M NaCl and then filtered using a sintered glass funnel. A 10-g sample of Trisacryl-sulphydryl was then equilibrated in 30 ml 0.1 M NaOAc, pH 6.0, and treated with 200 mg solid succinic anhydride. After shaking for 30 min, an additional 200 mg of anhydride was added to the suspension, and the capping reaction was allowed to proceed for a further 30 min. The beads were then equilibrated in 50 ml 0.1 M Tris, pH 8.5, to hydrolyze the thioester linkages. After 1 h, the support was washed with TE, pH 8.0, and stored at 4°C. The sulphydryl group concentration in the support was estimated by titrating with 5,5'-dithiobis(2-nitrobenzoic acid) and monitoring the release of 3-carboxylate-4-nitrothiophenolate at 412 nm.

Finally, the covalent attachment of 5'-bromoacetyl-derivatized oligonucleotides to sulphydryl Trisacryl was performed by the following procedure. The Trisacryl-sulphydryl support (1 g), obtained from the above reaction, was reduced with 30 ml 20 mM DTT in 0.05 M K_2HPO_4, pH 8.0, and 1 mM EDTA for 1 h. The support was then washed four times with 25 ml of 0.05 M K_2HPO_4, pH 8.0, 1 mM EDTA, followed by two washes with 25 ml of 0.1 M triethylammonium phosphate (TEAP), 1 mM EDTA, pH 9.0. Five nanomoles of bromoacetyl- derivatized oligonucleotide dissolved in 7 ml of 0.1 M TEAP, 1 mM EDTA, pH 9.0, was added to the support, and the tube was purged with N_2 and sealed. After overnight agitation on a rotary mixer, 200 mg of iodoacetic acid was added and the mixture was left at room temperature for 1 h. The beads were washed twice with 35 ml of 0.1 M Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA, and 0.1% SDS, four times with 45 ml of 0.1 M $Na_2P_2O_7$, pH 7.5, followed by two washes with 45 ml of TE, pH 8.0, and stored at 4°C.

EXAMPLE II

DETECTABLE LEVELS OF AMPLIFICATION OBSERVED WITH 3-ENZYME 3SR AMPLIFICATION OF A DNA TARGET

This example shows that detectable levels of amplification are observed with a 3-enzyme 3SR amplification of a DNA target.

Nucleic acids from 2.5 x 10^5 PBMC from both normal patients and patients with cystic fibrosis were extracted as in Example 1. The precipitated nucleic acids were pelleted by centrifugation. The pellet was drained, rinsed with 70% ethanol one time, dried and then resuspended in 100μl containing:

40mM	Tris-HCl, pH 8.1
10%	DMSO
10%	Glycerol

30mM $MgCl_2$
20mM KCl
4mM Spermidine
10mM Dithiothreitol
1mM each dATP, dGTP, dCTP and dTTP
7mM each rATP, rCTP, rGTP and rUTP
250ng each of oligonucleoctide primers

90-159
(5'AATTTAATACGACTCACTATAGGGAAATGCTTTGATGACGCTTCTG TA-3') (SEQ ID NO: 17)
90-161(5'-TTCACTTCTAATGATGATTATGGGAGAA-3') (SEQ ID NO: 18)

The samples were vortexed until the pellet was completely resuspended. As a control, water was used in place of the nucleic acid pellet in the above buffer.

The samples were heated at 100°C for 1 minute, cooled to 42°C for 1 minute and 10 units AMV reverse transcriptase (RT) (Life Science, Inc.) were added. The samples were incubated at 42°C for 15 minutes then heated to 100°C for 1 minute. Thirty units of AMV RT, 100 units T7 RNA polymerase (Stratagene) and 4 units E. coli Rnase H (Bethesda Research Labs) were added. The samples were incubated at 42°C. for 1 hour. The samples were then frozen at -20°C. The samples were then analyzed by bead-based sandwich hybridization using Oligobeads™ 90-294 (5'-GTTCTCAGTTTTCCTGGATTATGC-3') (SEQ ID NO: 19) and ^{32}P-labeled detection oligonucleotides 90-165 (5'- AAGAAAATATCATCTTTGGTGTTTCCT-3') (SEQ ID NO: 20) which detects the wild-type cystic fibrosis gene or 90-166 (5' AAAGAAAATATCATTGGTGTTTCCTA-3') (SEQ ID NO: 21) which detects a 3 base-deletion mutation within the cystic fibrosis gene.

In a typical bead-based sandwich hybridization (BBSH) procedure a 25mg aliquot of bead suspension is

added to a 2 ml micro-column (2S-GS, Isolab) and the TE solution is removed by forcing it through the column with a syringe. The target, in 20μl of TE, is added to the column, along with 10μl of 2x hybridization solution (20% dextran sulfate, 20x SSPE, 0.2% SDS) which had been warmed to 42°C. The micro-columns are vortexed and incubated with occasional agitation at 42°C for two hours. The beads are washed six times with 1ml each of 2x SSC which had been equilibrated at 42°C. Cerenkov counting of the columns and washes is used to determine the amount of target detected. Counter background is subtracted from all samples and the fm of target detected is calculated as follows:

$$\frac{\text{cpm on beads}}{(\text{cpm on beads} + \text{cpm washes})} \times \text{fm probe added}$$

rxn	Detection oligo	SEQ ID NO:	fm/ul 3SR
no target	90-165	20	0.016
2.5×10^5 wild-type pBMC w/w	90-165	20	0.113
no target	90-166	21	0.018
2.5×10^5 mutant pBMC Δ/Δ	90-166	21	2.243

EXAMPLE III

COMPARISON OF LEVELS OF AMPLIFICATION OBSERVED WITH THE 2-ENZYME 3SR REACTION AT 37°C WITH A PREFERRED REACTION MEDIUM OF THE PRESENT INVENTION AND WITH A PRIOR ART REACTION MEDIUM SUITABLE FOR 3-ENZYME 3SR REACTIONS

This Example shows that detectable levels of amplification are observed with the 2-enzyme 3SR reaction at 37°C with a preferred reaction medium of the present invention but not with a prior art reaction medium suitable for 3-enzyme 3SR reactions.

0.1 attomoles of HIV-1 RNA was amplified in 2-enzyme 3SR or 3-enzyme 3SR reactions at 37°C under

prior art reaction conditions and under improved reaction conditions of the invention. Under improved conditions, but not under prior art conditions, 2-enzyme 3SR amplification yielded a detectable amount of amplification product. Higher levels of amplification in the 3-enzyme reactions were observed with the improved reaction media, such as the preferred medium described below in this Example.

Each reaction solution contained 0.25 μg each of oligonucleotide primers 88-211* and 88-347*, 10 units AMV reverse transcriptase and 20 units T7 RNA polymerase. Total reaction volume was 100 μl. A "+" in the "Exogenously added RNAse H" column denotes the presence of 4 U E. coli RNAse H in the reaction medium, while a "+" in the "DMSO/PEG-8000" column denotes that the reaction medium was supplemented with 10% dimethylsulfoxide and 5% PEG-8000.

Prior Art 3SR Reaction Medium	Preferred 3SR Reaction Medium
40 mM Tris, pH 8.1	40 mM Tris, pH 8.1
20 mM $MgCl_2$	30 mM $MgCl_2$
25 mM NaCl	20 mM KCl
5 mM DTT	10 mM DTT
2 mM spermidine	4 mM spermidine
80 ug/ml BSA	Ø mg/ml BSA
1 mM dNTPs	1 mM dNTPs
4 mM rNTPs	7 mM rNTP

The reaction products were detected by bead-based sandwich hybridization (Example II) using Oligobeads™ derivatized with oligonucleotide #86-273 and oligonucleotide #87-81 as probe.

3SR Reactions on env Region at 37°C.

Buffer/Nucleotides	Exogenously added RNAse H	DMSO/PEG-8000	Fold Amp.
Prior art	+	-	$3.5X10^7$
Prior art	-	-	$< 10^4$
Prior art	-	+	$< 10^4$
Preferred	+	-	$1.7X10^8$
Preferred	-	-	$< 10^4$
Preferred	-	+	$1.1X10^5$

EXAMPLE IV

EFFECT OF SUPPLEMENTING THE PRESENTLY PREFERRED REACTION MEDIUM OF THE INVENTION WITH 10% DMSO, 10% GLYCEROL, AND/OR 5% POLYETHYLENE GLYCOL (PEG-8000) ON 2-ENZYME OR 3-ENZYME 3SR REACTIONS

This example demonstrates the effect of supplementing the presently preferred reaction medium of the invention (See Example III) with 10% DMSO, 10% Glycerol, and/or 5% polyethylene glycol (PEG-8000) on the level of amplification obtainable in 2-enzyme or 3-enzyme 3SR reactions.

The reaction conditions used for the 3SR reactions were the same as the "Preferred 3SR Reaction Medium" disclosed in Example III except that reactions were carried out at 42°C for 1 hour.

The 3SR reactions were carried out using 0.1 attomoles HIV-1 RNA as target and the primer pair 88-29/89-263* (approximately 400 bases apart). The products of amplification were detected by bead-based sandwich hybridization (Example II) using Oligobead™ derivatized with oligonucleotide 86-273 and oligonucleotide 87-81 as probe. As shown below, enhanced levels of amplification are obtained in the presence of 10% DMSO and 10% glycerol.

EFFECT OF ADDITIVES ON 3SR AMPLIFICATION ON THE ENV REGION OF HIV-1

Primers	RNase H	DMSO	Glycerol	PEG	Fold Amp.
88-299/89-263*	-	10%	-	5%	$1x10^5$
88-299/89-263*	-	10%	10%	-	$2x10^7$
88-211*/88-347*	4U	-	-	-	$2.8x10^8$
88-211*/88-347*	4U	-	10%	-	$2.0x10^8$
88-211*/88-347*	4U	10%	10%	-	$3.9x10^8$
88-211*/88-347*	-	-	-	-	N.D.
88-211*/88-347*	-	-	10%	-	N.D.
88-211*/88-347*	-	10%	10%	-	$1.3x10^7$

N.D.: No product detected

2-enzyme reactions: 10 U AMV RT, 20 U T7 RNA Polymerase

3-enzyme reactions: 30 U AMV RT, 100 U T7 RNA Polymerase, 4 U E. coli RNase H

EXAMPLE V

LEVELS OF 2-ENZYME 3SR AMPLIFICATION AND 3-ENZYME 3SR AMPLIFICATION IN THE PRESENCE AND ABSENCE OF 10% DMSO AND 5% PEG-8000 IN THE PREFERRED 3SR REACTION MEDIUM

This example compares the levels of 2-enzyme 3SR amplification and 3-enzyme 3SR amplification (0.1 attomoles of HIV-1 RNA target and primer pair 88-211*/88-347* in each case) in the presence and absence of 10% DMSO and 5% PEG-8000 in the preferred 3SR reaction medium. The products were detected by bead-based sandwich hybridization as in Example II using Oligobeads™ derivatized with oligonucleotide 86-273 and using oligonucleotide 87-81 as probe.

EFFECT OF ADDITIVES ON THE 2-ENZYME 3SR REACTION

Temp.	Enzymes	DMSO	PEG-8000	Fold Amp.
42°C	All 3	-	-	8.0×10^7
42°C	RT/T7	-	-	$< 10^4$
42°C	RT/T7	+	-	1.1×10^7
42°C	RT/T7	+	+	5.0×10^7
45°C	RT/T7	+	+	1.7×10^7

30 U AMV RT, 100 U T7 RNA Polymerase and, when present (i.e., "all 3"), 4 U E. coli RNase H were used in the reaction media.

EXAMPLE VI

2-ENZYME 3SR REACTIONS WITH REVERSE TRANSCRIPTASES FROM MOLONEY MURINE LEUKEMIA VIRUS, HIV-1 AND AVIAN MYELOBLASTOSIS VIRUS

This Example shows 2-enzyme 3SR reactions with reverse transcriptases (RTs) from Moloney murine leukemia virus (MMLV), HIV-1 and avian myeloblastosis virus (AMV). MMLV reverse transcriptase has a requirement for manganese ion to provide an effective amount of inherent RNAse H activity.

3SR System: env region 88-211*/88-347*
Comparison of Reverse Transcriptases
From Different Sources

RT		T7 Pol	RNase H	DMSO	Glycerol	$MnCl_2$	Fold Amp.
M-MLV	1000U	60U	4U	5%	-	-	$3x10^6$
M-MLV	1000U	60U	-	5%	-	1mM	$3x10^7$
M-MLV	1000U	60U	-	-	-	1mM	$2x10^7$
HIV	5ul[1/]	60U	-	-	-	-	$1x10^6$
HIV	10ul	60U	4U	-	-	-	$9x10^5$
AMV	30U	100U	4U	10%	10%	-	$4x10^8$
AMV	10U	20U	-	10%	10%	-	$1x10^8$

Reaction Time: 1 hour. Temp.: 42°C. Template: 0.1 amoles HIV RNA

1/ The specific activity of the HIV-1 reverse transcriptase preparation was unknown.

EXAMPLE VII

INCREASED AMPLIFICATION LEEVLS ACHIEVED IN 3-ENZYME 3SR REACTIONS, IN THE PRESENCE AND ABSENCE OF 5% PEG-8000/10% DMSO WITH INCUBATION AT 42°C or 45°C

This Example demonstrates increased amplification levels achieved in 3-enzyme 3SR reactions, in the presence and absence of 5% PEG-8000/10% DMSO with incubation at 42°C or 45°C.

0.1 attomoles of HIV-1 RNA was amplified in 3-enzyme 3SR reactions with 30 Units AMV reverse transcriptase, 100 Units T7 DNA-dependent RNA polymerase, 4 Units RNAse H, at 42° C for 1 hour in the preferred reaction medium disclosed in Example III using one of the following sets of env primer pairs: 88-211*/88-347* or 87-79/88-347*.

INFLUENCE OF REACTION TEMPERATURE ON AMPLFICATION OF ENV REGION OF HIV-1

		Fold Amplification	
Primer Pair	PEG-8000/DMSO	42°C	45°C.
88-211*/88-347*	-	4.4×10^7	8.4×10^6
	+	2.3×10^8	1.5×10^8
87-79/88-347*	-	4.5×10^7	1.5×10^7
	+	1.7×10^8	5.6×10^8

EXAMPLE VIII

EFFECT OF TEMPERATURE ON 3-ENZYME 3SR AMPLIFICATION IN THE PREFERRED REACTION MEDIUM IN THE PRESENCE AND ABSENCE OF 10% DMSO

This Example demonstrates the effect of temperature on 3-enzyme 3SR amplification in the preferred reaction medium (See Example III) in the presence and absence of 10% DMSO. The target was 0.1 attomoles of HIV-1 RNA. Each 100µl reaction mixture had 30 U AMV RT, 100 U T7 RNA Polymerase and 4 U E. coli RNase H.

Temperature Dependence of 3SR Reaction In Presence or Absence of DMSO

Primers 88-211* and 88-347* (env region)

Reaction Temp.	-DMSO	+DMSO
42°C	9.1×10^7	2.7×10^8
45°C	8.7x107	1.6×10^8
47°C	7.4×10^4	$<10^4$
50°C	$<10^4$	$<10^4$

EXAMPLE IX

EFFECT OF NUCLEOTIDE ALTERATIONS AT 5' AND 3' ENDS OF OLIGONUCLEOTIDE PRIMERS CONTAINING THE CONSENSUS SEQUENCE OF THE T7 PROMOTER

This example shows the effect of nucleotide alterations at 5' and 3' ends of oligonucleotide primers containing the consensus sequence of the T7 promoter.

3SR reactions were performed in 50μl containing 0.05 attomoles (~1,000 molecules) of HIV-1 RNA target, 30U T7 RNA polymerase, 2U E. coli RNAseH, 15U AMV RT, 40mM Tris, pH 8.1, 30mM MgCl2, 20mM KCl, 10mM DTT, 4mM spermidine, 1mM dXTP, 7mM rXTPs, and 0.125μg of oligonucleotide primers for 1-2 hours at 42°C. The same companion primer, 89-255 (5'TTATTGTGCCCCGGCTGGTTTTGCGATTCTA3') (SEQ ID NO: 14) (Ratner et al. 1985) was used for each primer listed in this table. The listed primers encode the cannonical 17 nt T7-promoter sequence (except #90-206, having the 5' nucleotide thereof deleted) and have varying lengths and compositions respecting the 5' and 3' flanking sequences to the consensus 17 nt T7-promoter sequence. The RNA product made is 214 nucleotides long. Amplified targets were quantitated using beadbased sandwich hybridization (BBSH) employing 25mg of beads containing capture oligonucleotide 86-273 and 100 fmoles of ^{32}P-labeled detection of oligonucleotides 87-81 (Guatelli et al. 1990). All amplification values, except for 88-347*, are the average of two 3SR reactions and each of these amplification reaction was analyzed by duplicate BBSH reactions. The 3SR amplifications performed with 88-347* represent the average of six 3SR reactions.

EFFECT ON 3SR AMPLIFICATIONS OF NUCLEOTIDE AT THE TRANSCRIPTION INITIATION SEQUENCE OF OLIGONUCLEOTIDE PRIMERS CONTAINING T7 PROMOTER SEQUENCES

SEQ ID NO:	Oligo-Nucleotide	Length (nt) 5' -17	Sequence[1] +1 3'	Fold Amp.
2	88-347	56	AATTTAATACGACTCACTATAGGGATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2x10^8$
22	90-426	56	AATTTAATACGACTCACTATAGAAATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.4x10^9$
23	90-199	56	AATTTAATACGACTCACTATAGGTATGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.1x10^9$
24	90-200	56	AATTTAATACGACTCACTATAGGAATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.2x10^9$
25	90-201	56	AATTTAATACGACTCACTATAGGCATGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.8x10^9$
26	90-202	55	AATTTAATACGACTCACTATAGG ATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.0x10^9$
27	90-203	54	AATTTAATACGACTCACTATAG ATGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.5x10^9$
28	90-204	53	AATTTAATACGACTCACTATAG TGTACTATTATGGTTTTAGCATTGTCTGTGA	$1.9x10^9$
29	90-430	52	AATTTAATACGACTCACTATA TGTACTATTATGGTTTTAGCATTGTCTGTGA	$2.8x10^9$

1/ Coding strand sequence is displayed. The underlined sequence corresponds to the cannonical 17 nt T7-promoter sequence and the initiation of RNA transcription is denoted by +1. The sequence GGGA is from the T7 sequence; the +5 nucleotides onward is the HIV-1 sequence. Only the 5' end portion of each oligonucleotide is presented; subsequent sequences are identical for each oligonucleotide. Oligonucleotides are aligned to display differences among the primers.

EXAMPLE X

EFFECT OF VARIOUS COMBINATIONS OF ADDITIVES ON THE AMPLIFICATION OF A REGION OF THE POL GENE FROM HIV-1

This example demonstrates the effect of various combinations of additives on the amplification of a 707-base region of the pol gene from HIV-1. Reactions were performed at 42°C for two hours with 0.1 attomoles of HIV-1 RNA as the target and 90-249 (sense) 5'-GAAAAAATAAAAGCATTAGTAGA-3' (SEQ ID NO: 30) and 89-391* (antisense) 5'-AATTTAATACGACTCACTATAGGGATTTCCCCACTAACTTCTGTATGTCATTGACA-3' (SEQ ID NO: 31) as the priming oligonucleotides. Three-enzyme reactions contained 30 U AMV RT, 100 U T7 RNA polymerase and 2 U E.Coli RNase H. Two-enzyme reactions contained 10 U AMV RT and 20 U T7 RNA polymerase. The probe and Oligobead™ sequences were 89-534 5'-AGGATCTGACTTAGAAATAGGGCAGCA-3' (SEQ ID NO: 32) and 89-419 5'-AGAACTCAAGACTTCTGGGAAGTTC-3' (SEQ ID NO: 33), respectively.

EFFECT OF ADDITIVE COMBINATION ON 2- AND 3-ENZYME AMPLIFICATION OF A REGION OF THE POL REGION OF HIV-1

Additives	Fold Amplification	
	3-enzyme	2-enzyme
none	7.9×10^4	n.d.
10%DMSO/10%Glycerol	8.4×10^6	1.2×10^5
10%DMSO/5% PEG-8000	4.6×10^4	n.d.
10%DMSO/15% Sorbitol	7.0×10^6	1.3×10^6

n.d. = no product detected by bead-based sandwich hydridization

While the invention has been described with some specificity, modifications apparent to those with

ordinary skill in the art may be made without departing from the spirit of the invention.

Various features of the invention are set forth in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fahy, Eoin D.
Kwoh, Deborah Y.
Gingeras, Thomas R.
Guatelli, John C.
Whitfield, Kristina M.

(ii) TITLE OF INVENTION: Nucleic Acid Amplification by Two-Enzyme Self-Sustained Sequence Replication

(iii) NUMBER OF SEQUENCES: 33

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fitch, Even, Tabin & Flannery
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(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60603-4277

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Feder, Scott B.
(C) REFERENCE/DOCKET NUMBER: 50101

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312-372-7842
(B) TELEFAX: 312-372-7848

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-425

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGTAATTTAA TACGACTCAC TATAGGGATG TACTATTATG GTTTTAGCAT TGTCTGTGA 59

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 88-347

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATTTAATAC GACTCACTAT AGGGATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-578

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATTTAATAC GACTCACTAT AGGGATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-575

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTTTAATAC GACTCACTAT AGGGATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-577

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGTTAATAC GACTCACTAT AGGGATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-574

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCTAATAC GACTCACTAT AGGGATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-247

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATTTAATACG ACTCACTATA GGGATGTACT ATTATGGTTT TAGCATTGTC TGTGA 55

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-248

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTAATACGA CTCACTATAG GGATGTACTA TTATGGTTTT AGCATTGTCT GTGA 54

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-576

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTTAATACGA CTCACTATAG GGATGTACTA TTATGGTTTT AGCATTGTCT GTGA 54

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-579

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCTAATACGA CTCACTATAG GGATGTACTA TTATGGTTTT AGCATTGTCT GTGA 54

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-249

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTAATACGAC TCACTATAGG GATGTACTAT TATGGTTTTA GCATTGTCT GTGA 53

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-205

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TAATACGACT CACTATAGGG ATGTACTATT ATGGTTTTAG CATTGTCTG TGA 52

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-206

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATACGACTC ACTATAGGGA TGTACTATTA TGGTTTTAGC ATTGTCTGT GA 51

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 89-255

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTATTGTGCC CCGGCTGGTT TTGCGATTCT A 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: T7 Native Promoter with TIS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTTAATAC GACTCACTAT AGGGA 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 88-297

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGGCCTAATT CCATGTGTAC ATTGTACTGT 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-159

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AATTTAATAC GACTCACTAT AGGGAAATGC TTTGATGACG CTTCTGTA 48

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-161

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTCACTTCTA ATGATGATTA TGGGAGAA 28

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTTCTCAGTT TTCCTGGATT ATGC 24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-165

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGAAAATAT CATCTTTGGT GTTTCCT 27

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-166

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AAAGAAAATA TCATTGGTGT TTCCTA 26

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-426

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AATTTAATAC GACTCACTAT AGAAATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-199

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATTTAATAC GACTCACTAT AGGTATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-200

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AATTTAATAC GACTCACTAT AGGAATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-201

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AATTTAATAC GACTCACTAT AGGCATGTAC TATTATGGTT TTAGCATTGT CTGTGA 56

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-202

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AATTTAATAC GACTCACTAT AGGATGTACT ATTATGGTTT TAGCATTGTC TGTGA 55

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-203

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AATTTAATAC GACTCACTAT AGATGTACTA TTATGGTTTT AGCATTGTCT GTGA 54

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-204

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AATTTAATAC GACTCACTAT AGTGTACTAT TATGGTTTTA GCATTGTCTG TGA 53

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-430

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AATTTAATAC GACTCACTAT ATGTACTATT ATGGTTTTAG CATTGTCTGT GA 52

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 90-249

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAAAAAATAA AAGCATTAGT AGA 23

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 89-391

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATTTAATAC GACTCACTAT AGGGATTTCC CCACTAACTT CTGTATGTCA TTGACA 56

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 89-534

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AGGATCTGAC TTAGAAATAG GGCAGCA 27

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: 89-419

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AGAACTCAAG ACTTCIGGGA AGTTC 25

WE CLAIM:

1. A method for 3SR amplification of a target RNA segment of a target RNA molecule which segment comprises a 5'-subsegment, which includes a 5'-terminal nucleotide and extends at least 9 nucleotides in the 3'-direction from the 5'-terminal nucleotide of the target segment, and a 3'-subsegment, which does not overlap the 5'-subsegment and which includes a 3'-terminal nucleotide and extends at least 9 nucleotides in the 5'-direction from the 3'-terminal nucleotide of the target segment, which method comprises incubating in a reaction medium:

(a) (1) a first DNA primer which is a single stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said first primer being of the same length as the 3'-subsegment of the target segment and having a sequence sufficiently complementary to that of the 3'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence of the target segment is the template, and (2) a second DNA primer, which is a single-stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said second primer being of the same length as the 5'-subsegment of the target segment and having a sequence sufficiently homologous to that of the 5'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence complementary to that of the target segment is the template, provided that at least one of said primers further comprises a promoter-providing subsegment, which comprises the sense strand of a first promoter, said sense strand being joined to the first subsegment of the primer, which

comprises said promoter-providing segment, operably for transcription from said first promoter of a cDNA comprising the extension products of said two primers, and provided further that, where said first primer lacks such a promoter-providing subsegment, then the 5'-terminal nucleotide of said 5'-subsegment of said target RNA segment is the 5'-terminal nucleotide of the target RNA molecule;

(b) at least two enzymes which exhibit in said reaction medium DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, RNAse H activity and a DNA-dependent RNA polymerase, said DNA-dependent RNA polymerase in said reaction medium, being capable of catalyzing transcription from said first promoter; and

(c) nucleoside triphosphates required as substrates for the DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and DNA-dependent RNA polymerase activities;

wherein said incubation occurs in a range of temperatures at which said enzymes in said reaction medium are active in providing said DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, RNAse H, and DNA-dependent RNA polymerase activities.

2. A method according to Claim 1 wherein the target segment is fewer than about 1500 nucleotides in length, wherein both the 5'-subsegment and the 3'-subsegment of the target segment have about 15-50 nucleotides, and wherein said incubation occurs at about 40° C.

3. A method according to Claim 2 wherein the reverse transcriptase is a retroviral reverse transcriptase.

4. A method according to Claim 3 wherein the reaction medium comprises a reverse transcriptase enzyme, an RNAse H enzyme and a DNA dependent RNA polymerase.

5. A method according to Claim 4 wherein the RNAse H enzyme is E. coli RNAse H.

6. A method according to Claim 5 wherein the DNA-dependent RNA polymerase activity is provided by the RNA polymerase of a bacteriophage selected from the group consisting of T7, T3 and SP6.

7. A method according to Claim 6 wherein the RNA target segment is less than about 200 nucleotides in length.

8. A method according to Claim 7 wherein after the incubation to amplify the target RNA segment, said target RNA segment or the RNA segment with the sequence complementary to that of said target RNA segment is detected in a nucleic acid probe hybridization assay.

9. A method for amplifying a target RNA segment which comprises a 5'-subsegment, which includes and extends at least 9 nucleotides in the 3'-direction from the 5'-terminal nucleotide of the target segment, and a 3'-subsegment, which does not overlap the 5'-subsegment and which includes and extends at least 9 nucleotides in the 5'-direction from the 3'-terminal nucleotide of the target segment, which method comprises incubating in an aqueous solution comprising the target RNA segment:

(a) (1) a first DNA primer which is a single stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said first primer being of the same length as the 3'-subsegment of the target segment and having a sequence sufficiently complementary to that of the 3'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence of the target segment is the template, and (2) a second DNA primer, which is a single-stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said second primer being of the same length as the 5'-subsegment of the target segment and having a

sequence sufficiently homologous to that of the 5'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence complementary to that of the target segment is the template, provided that at least one of said primers further comprises a promoter-providing subsegment, which comprises the sense strand of a first promoter, said sense strand being joined to the first subsegment of the primer, which comprises said promoter-providing segment, operably for transcription from said first promoter of a cDNA comprising the extension products of said two primers, and provided further that, where said first primer lacks such a promoter-providing subsegment, then the 5'-terminal nucleotide of said 5'-subsegment of said target RNA segment is the 5'-terminal nucleotide of the target RNA molecule;

(b) (1) a reverse transcriptase which exhibits in said reaction medium DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and a high sensitivity amplification-effective amount of RNAse H activity, and (2) a DNA-dependent RNA polymerase which, in said reaction medium, catalyzes transcription from said first promoter; and

(c) nucleoside triphosphates required as substrates for the DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and DNA-dependent RNA polymerase activities;

said incubation occurring in a range of temperatures at which said enzymes in said solution are active in providing said DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, RNAse H, and DNA-dependent RNA polymerase activities.

10. A method according to Claim 9 wherein the reaction medium comprises 20-40 mM $MgCl_2$, 1-25 mM KCl, 1-20 mM dithiothreitol, 1-10 mM spermidine, 1-7 mM rNTPs, 0.1-2 mM dNTPs, and an effective amount of buffer to

maintain the reaction medium at about pH 8.

11. A method according to Claim 10 wherein the target segment is fewer than about 1500 nucleotides in length, wherein both the 5'-subsegment and the 3'-subsegment of the target segment have about 15-50 nucleotides, and wherein said incubation occurs between about 37°C and about 47°C.

12. A method according to Claim 11 wherein the reverse transcriptase is a retroviral reverse transcriptase.

13. A method according to Claim 12 wherein the concentration of rNTPs is about 6 mM and wherein the reaction medium further comprises between about 1 and about 25 weight percent of at least one compound from the group consisting of (i) a C1-C10 alcohol; (ii) a sugar alcohol of the formula $HOCH_2(CHOH)_xCH_2OH$, wherein x is 0-20; (iii) a polyethylene glycol compound of the formula $H(OCH_2-CH_2)_nOH$, wherein n is 2-600; (iv) a sugar from the group of mono-, di- and tri- saccharides; and (v) a sulfoxide compound of the formula $R_1-(SO)-R_2$, wherein R_1 and R_2 are independently C_1 - C_4 alkyl, wherein R_1 and R_2 may be joined as part of a saturated cyclic compound.

14. A method according to Claim 13 wherein said at least one compound is selected from the group of sorbitol, glycerol, ethanol, sucrose, polyethylene glycol and dimethylsulfoxide.

15. A method according to Claim 14 wherein the DNA-dependent RNA polymerase activity is provided by the RNA polymerase of a bacteriophage selected from the group consisting of T7, T3 and SP6.

16. A method according to Claim 15 wherein the reaction medium comprises 30 mM $MgCl_2$, 20 mM KCl, 10 mM dithiothreitol, 4 mM spermidine, 6 mM rNTPs, 1 mM dNTPs.

17. A method according to Claim 16 wherein the sequence of the first subsegment of the first primer is exactly complementary to the sequence of the 3'-subsegment of the target segment and wherein the

sequence of the first subsegment of the second primer is the same as the sequence of the 5'-subsegment of the target segment.

18. A method according to Claim 17 wherein, after the incubation to amplify the target RNA segment, said target RNA segment or the RNA segment with the sequence complementary to that of said target RNA segment is detected in a nucleic acid probe hybridization assay.

19. A method according to Claim 12 wherein the reverse transcriptase is AMV reverse transcriptase and the reaction media further comprises between about 1 and about 25 weight percent of a sulfoxide compound of the formula R_1-(SO)-R_2, wherein R_1 and R_2 are independently C_1 - C_4 alkyl, wherein R_1 and R_2 can be joined as part of a saturated cyclic compound.

20. A method according to Claim 19 wherein the reaction medium comprises dimethylsulfoxide.

21. A method according to Claim 20 wherein the concentration of rNTPs is about 6 mM and wherein the reaction medium further comprises between about 1 and about 25 weight percent of at least one compound from the group consisting of (i) a C1-C10 alcohol; (ii) a sugar alcohol of the formula $HOCH_2(CHOH)_xCH_2OH$, wherein x is 0-20; (iii) a polyethylene glycol compound of the formula $H(OCH_2-CH_2)_nOH$, wherein n is 2-600; and (iv) a sugar from the group of mono-, di- and tri saccharides.

22. A method according to Claim 21 wherein said compound is selected from the group of sorbitol, glycerol, ethanol, sucrose and polyethylene glycol.

23. A method according to Claim 22 wherein the DNA-dependent RNA polymerase activity is provided by the RNA polymerase of a bacteriophage selected from the group consisting of T7, T3 and SP6.

24. A method according to Claim 23 wherein the aqueous solution comprises 30 mM $MgCl_2$, 20 mM KCl, 10 mM dithiothreitol, 4 mM spermidine, 6 mM rNTPs, 1 mM dNTPs.

25. A method according to Claim 24 wherein the

sequence of the first subsegment of the first primer is exactly complementary to the sequence of the 3'-subsegment of the target segment and wherein the sequence of the first subsegment of the second primer is the same as the sequence of the 5'-subsegment of the target segment.

26. A method according to Claim 25 wherein wherein the RNA target segment has a length of at least 50 nucleotides and the target hybridizing segments of the two DNA primers are each about 15 nucleotides in length.

27. A method according to Claim 26 wherein, after the incubation to amplify the target RNA segment, said target RNA segment or the RNA segment with the sequence complementary to that of said target RNA segment is detected in a nucleic acid probe hybridization assay.

28. A method according to Claim 12 wherein the reverse transcriptase is Moloney murine leukemia virus reverse transcriptase and wherein the aqueous solution comprises between about 0.1 mM to about 10 mM manganese ions.

29. A method according to Claim 28 wherein the concentration of rNTPs is about 6 mM and wherein the reaction medium further comprises between about 1 and about 25 weight percent of at least one compound from the group consisting of (i) a C1-C10 alcohol; (ii) a sugar alcohol of the formula $HOCH_2(CHOH)_xCH_2OH$, wherein x is 0-20; (iii) a polyethylene glycol compound of the formula $H(OCH_2-CH_2)_nOH$, wherein n is 2-600; (iv) a sugar from the group of mono-, di- and trisaccharides; and (v) a sulfoxide compound of the formula $R_1-(SO)-R_2$, wherein R_1 and R_2 are independently C_1 - C_4 alkyl, wherein R_1 and R_2 can be joined as part of a saturated cyclic compound.

30. A method according to Claim 29 wherein said compound is selected from the group of sorbitol, glycerol, ethanol, sucrose, polyethylene glycol and dimethylsulfoxide.

31. A method according to Claim 30 wherein the

DNA-dependent RNA polymerase activity is provided by the RNA polymerase of a bacteriophage selected from the group consisting of T7, T3 and SP6.

32. A method according to Claim 31 wherein the aqueous solution comprises 30 mM $MgCl_2$, 20 mM KCl, 10 mM dithiothreitol, 4 mM spermidine, 6 mM rNTPs, 1 mM dNTPs.

33. A method according to Claim 32 wherein the RNA target segment has a length of at least 50 nucleotides and the target hybridizing segments of the two DNA primers are each about 15 nucleotides in length.

34. A method according to Claim 33 wherein the sequence of the first subsegment of the first primer is exactly complementary to the sequence of the 3'-subsegment of the target segment and wherein the sequence of the first subsegment of the second primer is the same as the sequence of the 5'-subsegment of the target segment.

35. A method according to Claim 34 wherein, after the incubation to amplify the target RNA segment, said target RNA segment or the RNA segment with the sequence complementary to that of said target RNA segment is detected in a nucleic acid probe hybridization assay.

36. A method according to Claim 12 wherein the reaction medium further comprises E. coli RNAse H.

37. A method according to Claim 36 wherein the reaction medium further comprises a sulfoxide compound of the formula R_1-(SO)-R_2, wherein R_1 and R_2 are independently C_1 - C_4 alkyl, wherein R_1 and R_2 can be joined as part of a saturated cyclic compound.

38. A method according to Claim 37 wherein the sulfoxide compound is DMSO.

39. A method according to Claim 38 wherein the concentration of rNTPs is about 6 mM and wherein the reaction medium further comprises between about 1 and about 25 weight percent of at least one compound from the group consisting of (i) a C1-C10 alcohol; (ii) a sugar alcohol of the formula $HOCH_2(CHOH)_xCH_2OH$, wherein x is

0-20; (iii) a polyethylene glycol compound of the formula $H(OCH_2-CH_2)_nOH$, wherein n is 2-600; (iv) a sugar from the group of mono-, di- and tri saccharides.

40. A method according to Claim 39 wherein said compound is selected from the group of sorbitol, glycerol, ethanol, sucrose, and polyethylene glycol.

41. A method according to Claim 40 wherein the DNA-dependent RNA polymerase activity is provided by the RNA polymerase of a bacteriophage selected from the group consisting of T7, T3 and SP6.

42. A method according to Claim 41 wherein the aqueous solution comprises 30 mM $MgCl_2$, 20 mM KCl, 10 mM dithiothreitol, 4 mM spermidine, 6 mM rNTPs, 1 mM dNTPs.

43. A method according to Claim 42 wherein the reverse transcriptase is selected from the group of AMV reverse transcriptase, MMLV reverse transcriptase and HIV-1 reverse transcriptase.

44. A method according to Claim 43 wherein the reverse transcriptase is AMV reverse transcriptase, wherein the RNA target segment has a length greater than about 400 nucleotides and wherein the reaction medium further comprises 10% DMSO and 15% sorbitol.

45. A method according to Claim 44 wherein the sequence of the first subsegment of the first primer is exactly complementary to the sequence of the 3'-subsegment of the target segment and wherein the sequence of the first subsegment of the second primer is the same as the sequence of the 5'-subsegment of the target segment.

46. A method according to Claim 45 wherein, after the incubation to amplify the target RNA segment, said target RNA segment or the RNA segment with the sequence complementary to that of said target RNA segment is detected in a nucleic acid probe hybridization assay.

47. A method according to Claim 24 wherein the first DNA primer comprises said promoter-providing segment and the second primer lacks a promoter-providing

segment.

48. A method according to Claim 47 wherein the reaction medium is supplemented with 10% DMSO and 15% sorbitol and wherein the RNA target segment has a length less than about 700 nucleotides.

49. A method according to Claim 48 wherein the promoter-providing segment is from the T7 promoter.

50. A method according to Claim 49 wherein the sequence of the first subsegment of the first primer is exactly complementary to the sequence of the 3'-subsegment of the target segment and wherein the sequence of the first subsegment of the second primer is the same as the sequence of the 5'-subsegment of the target segment.

51. A method according to Claim 50 wherein the RNA target segment has a length of at least 50 nucleotides and the target hybridizing segments of the two DNA primers are each about 15 nucleotides in length.

52. A method according to Claim 24 wherein the first primer comprises a first promoter-providing subsegment, to provide a first promoter to initiate transcription of a cDNA comprising the extension products of the two primers, and the second primer comprises a second promoter-providing subsegment, to provide a second promoter to initiate transcription of a cDNA comprising the extension products of the two primers, said first promoter, in the reaction medium, being recognized by a first DNA-dependent RNA polymerase for catalysis of transcription and said second promoter, in the reaction medium, being recognized by a second DNA-dependent RNA polymerase for catalysis of transcription, said first and second DNA-dependent RNA polymerases being the same or different; and wherein the reaction medium comprises said second DNA-dependent RNA polymerase.

53. A method according to Claim 52 wherein said first DNA-dependent RNA polymerase is different from said second DNA-dependent RNA polymerase, wherein, in the

reaction medium, said second DNA-dependent RNA polymerase but not said first DNA-dependent RNA polymerase recognizes said second promoter; and wherein the reaction medium comprises said second DNA-dependent RNA polymerase.

54. A method according to Claim 53 wherein the sequence of the first subsegment of the first primer is exactly complementary to the sequence of the 3'-subsegment of the target segment and wherein the sequence of the first subsegment of the second primer is the same as the sequence of the 5'-subsegment of the target segment.

55. A method according to Claim 54 wherein the RNA target segment has a length of at least 50 nucleotides and the target hybridizing segments of the two DNA primers are each about 15 nucleotides in length.

56. A kit useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, which kit consists essentially of:

(a) (1) a first DNA primer which is a single stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said first primer being of the same length as the 3'-subsegment of the target segment and having a sequence sufficiently complementary to that of the 3'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence of the target segment is the template, and (2) a second DNA primer, which is a single-stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said second primer being of the same length as the 5'-subsegment of the target segment and having a sequence sufficiently homologous to that of the 5'-subsegment of the target segment to prime, in the

reaction medium, a primer extension reaction in which a nucleic acid with the sequence complementary to that of the target segment is the template, provided that at least one of said primers further comprises a promoter-providing subsegment, which comprises the sense strand of a first promoter, said sense strand being joined to the first subsegment of the primer, which comprises said promoter-providing segment, operably for transcription from said first promoter of a cDNA comprising the extension products of said two primers, and provided further that, where said first primer lacks such a promoter-providing subsegment, then the 5'-terminal nucleotide of said 5'-subsegment of said target RNA segment is the 5'-terminal nucleotide of the target RNA molecule;

(b) (1) a reverse transcriptase which exhibits in said reaction medium DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and an amplification-effective amount of RNAse H activity, and (2) a DNA-dependent RNA polymerase which, in said reaction medium, catalyzes transcription from said first promoter; and

(c) nucleoside triphosphates required as substrates for the DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and DNA-dependent RNA polymerase activities.

57. A kit useful for the detection of at least one specific RNA target sequence in a sample containing nucleic acid, which kit comprises:

an aqueous medium comprising from about 20 to 40 mM $MgCl_2$; about 1 to 25 mM KCl; about 1 to 20 mM dithiothreitol; about 1 to 10 mM spermidine; about 1 to 8 mM rNTPs; about 0.1 to 2 mM dNTPs; and an amount of buffer effective to maintain the medium at about pH 8.

58. A kit according to Claim 57 wherein the reaction medium comprises 30 mM $MgCl_2$, 20 mM KCl, 10 mM dithiothreitol, 4 mM spermidine, 6 mM rNTPs, 1 mM dNTPs,

and 40 mM Tris·HCl and wherein the pH is about pH 8.1.

59. A pair of DNA primers for amplifying, in a transcription based amplification system, a target nucleic acid segment which comprises a 5'-subsegment, which includes and extends at least 9 nucleotides in the 3'-direction from the 5'-terminal nucleotide of the target segment, and a 3'-subsegment, which does not overlap the 5'-subsegment and which includes and extends at least 9 nucleotides in the 5'-direction from the 3'-terminal nucleotide of the target segment, said DNA primers comprising:

(1) a first DNA primer which is a single stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said first primer being of the same length as the 3'-subsegment of the target segment and having a sequence sufficiently complementary to that of the 3'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence of the target segment is the template, and

(2) a second DNA primer, which is a single-stranded DNA which comprises at its 3'-end a first subsegment having a 3'-terminal nucleotide and extending at least 9 nucleotides in the 5'-direction, said first subsegment of said second primer being of the same length as the 5'-subsegment of the target segment and having a sequence sufficiently homologous to that of the 5'-subsegment of the target segment to prime, in the reaction medium, a primer extension reaction in which a nucleic acid with the sequence complementary to that of the target segment is the template, provided that at least one of said primers further comprises a promoter-providing subsegment, which promoter-providing subsegment comprises (i) at its 5'-end a DNA segment having between one and ten nucleotides, said one to ten nucleotide segment being joined through a single phosphodiester linkage to (ii)

the 5'-nucleotide of a polymerase binding segment of a sense strand of a first promoter, said polymerase binding segment having the same length as the consensus sequence of said sense strand of said first promoter being joined to the first subsegment of the primer, which comprises said promoter-providing segment, operably for transcription from said first promoter of a cDNA comprising the extension products of said two primers.

60. A pair of DNA primers according to Claim 59 wherein the promoter-providing segment further comprises a transcription initiation segment having between one and four nucleotides, said transcription initiation sequence being joined through a single phosphodiester linkage to the 3'-nucleotide of the promoter consensus sequence.

61. A pair of DNA primers according to Claim 60 wherein the transcription initiation sequence is 5'-GAAA-3'.

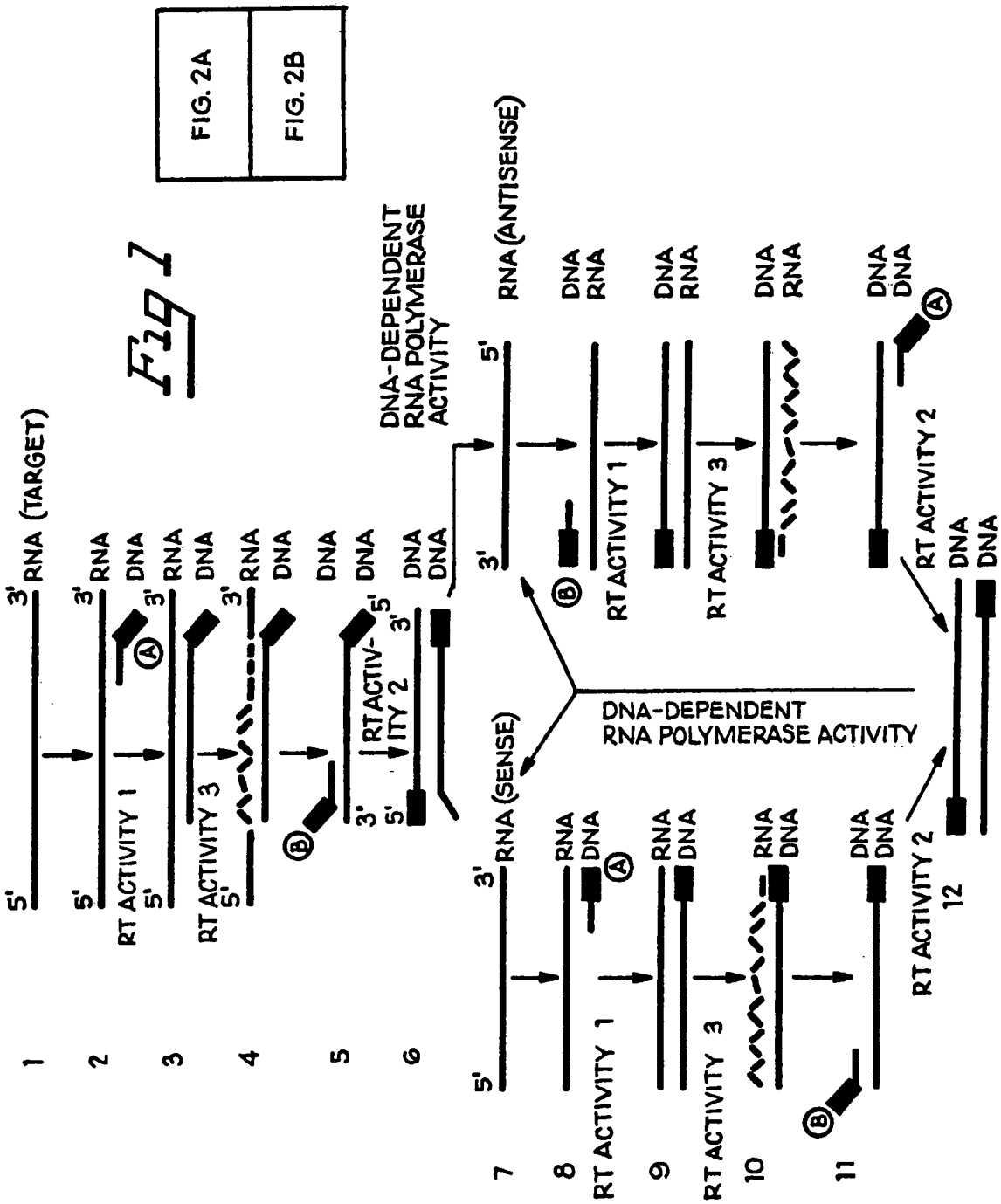
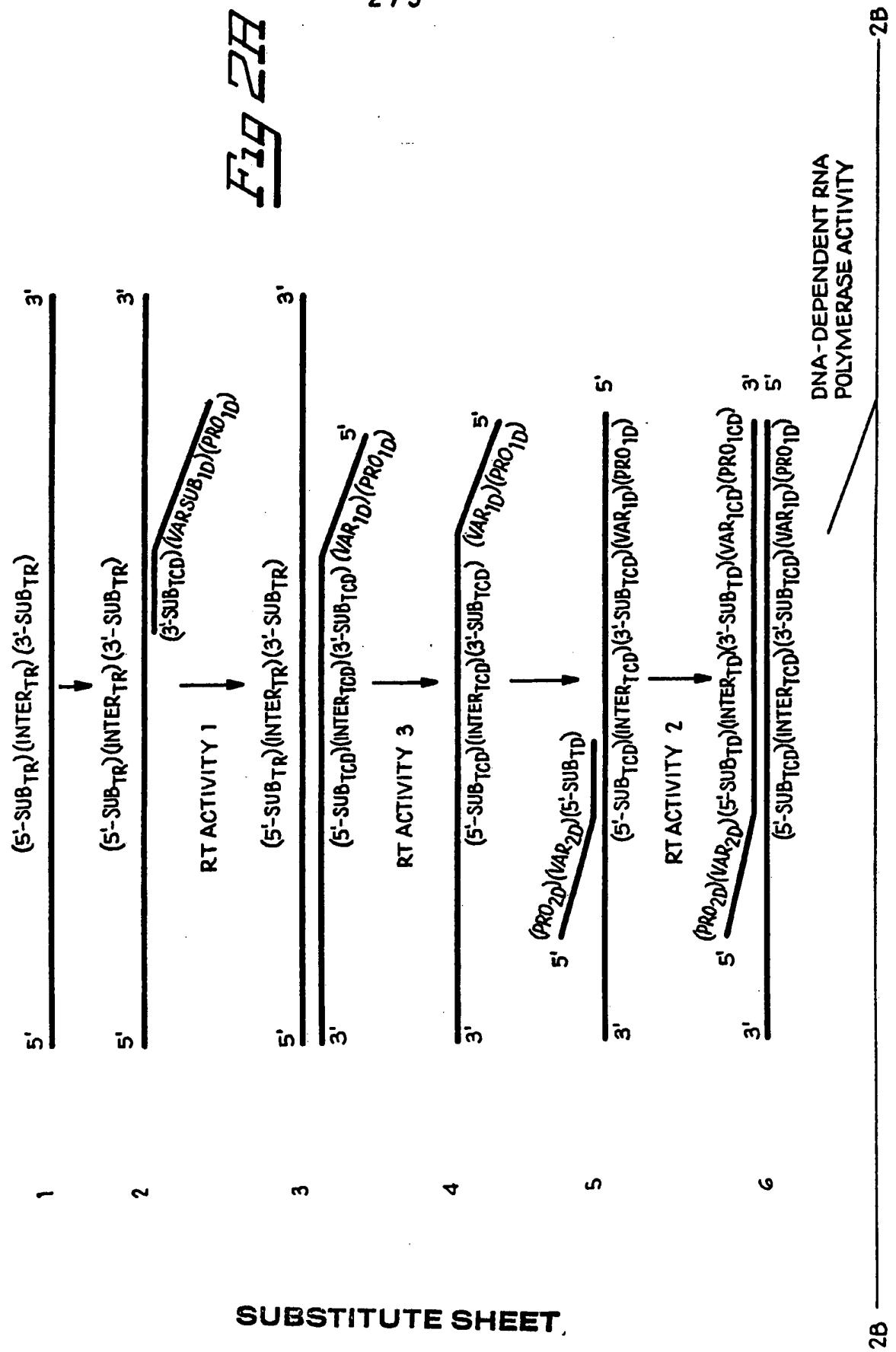


FIG. 2A
FIG. 2B
Fig 1
RNA (TARGET)
RT ACTIVITY 1
RT ACTIVITY 3
RT ACTIV-ITY 2
DNA-DEPENDENT RNA POLYMERASE ACTIVITY
RNA (ANTISENSE)
RNA (SENSE)
DNA-DEPENDENT RNA POLYMERASE ACTIVITY
RT ACTIVITY 2
RNA
DNA
5'
3'
A
B
1
2
3
4
5
6
7
8
9
10
11
12

Fig 2A


1
2
3
4
5
6
5'-SUB$_{TR}$)(INTER$_{TR}$)(3'-SUB$_{TR}$)
(5'-SUB$_{TR}$)(INTER$_{TR}$)(3'-SUB$_{TR}$)
(3'-SUB$_{TCD}$)(VARSUB$_{1D}$)(PRO$_{1D}$)
RT ACTIVITY 1
(5'-SUB$_{TR}$)(INTER$_{TR}$)(3'-SUB$_{TR}$)
(5'-SUB$_{TCD}$)(INTER$_{TCD}$)(3'-SUB$_{TCD}$)(VAR$_{1D}$)(PRO$_{1D}$)
RT ACTIVITY 3
(5'-SUB$_{TCD}$)(INTER$_{TCD}$)(3'-SUB$_{TCD}$)(VAR$_{1D}$)(PRO$_{1D}$)
(PRO$_{2D}$)(VAR$_{2D}$)(5'-SUB$_{TD}$)
(5'-SUB$_{TCD}$)(INTER$_{TCD}$)(3'-SUB$_{TCD}$)(VAR$_{1D}$)(PRO$_{1D}$)
RT ACTIVITY 2
(PRO$_{2D}$)(VAR$_{2D}$)(5'-SUB$_{TD}$)(INTER$_{TD}$)(3'-SUB$_{TD}$)(VAR$_{1CD}$)(PRO$_{1CD}$)
(5'-SUB$_{TCD}$)(INTER$_{TCD}$)(3'-SUB$_{TCD}$)(VAR$_{1D}$)(PRO$_{1D}$)
DNA-DEPENDENT RNA POLYMERASE ACTIVITY
2B
2B

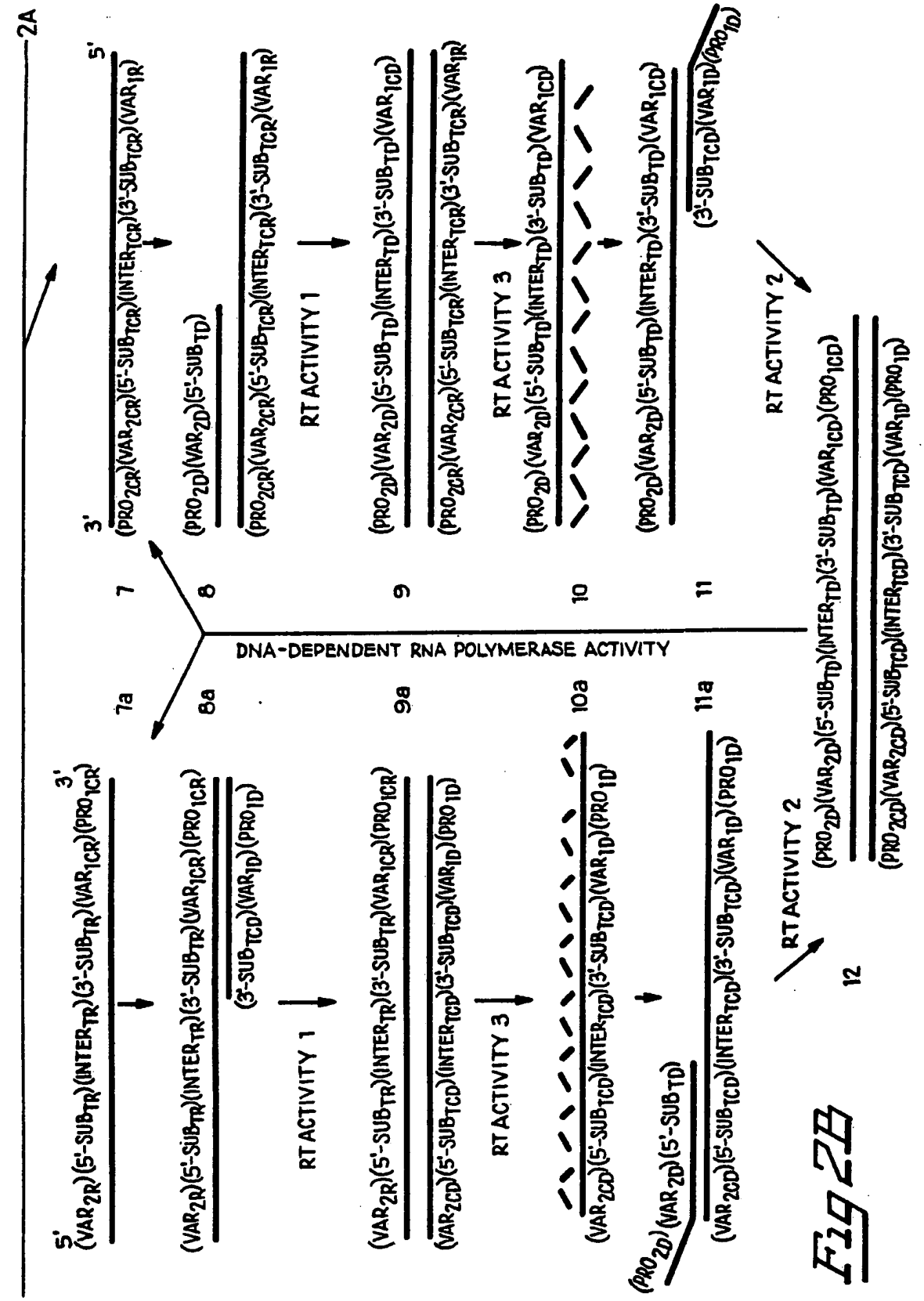

2A
2A
DNA-DEPENDENT RNA POLYMERASE ACTIVITY
RT ACTIVITY 1
RT ACTIVITY 3
RT ACTIVITY 2
RT ACTIVITY 1
RT ACTIVITY 3
RT ACTIVITY 2
7
7a
8
8a
9
9a
10
10a
11
11a
12

Fig 2B

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08488

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 19/34; C12Q 1/68; C07H 15/12; C07H 17/00

U.S.Cl.: 435/91; 435/6; 536/27; 435/810

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	435/6; 435/91; 536/27; 435/810

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS CAS Data Bases

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	US, A, 4,800,159 (Mullis et al) 24 January 1989, see column 29, example 9.	59, 60, 61 1-4, 9-18, 28-35, 56, 57, 58,
Y	EP, A, 0,329, 822 (Davey et al) 30 August 1989, see abstract.	1-18, 28-36, 56-61
Y	EP, A, 0,373,960 (Gingeras et al) 20 June 1990, see abstract.	1-18, 28-36, 56-61
Y	Proc. Natl. Acad. Sci., Volume 87, issued March 1990, Guatelli et al. "Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retrovical replication", pages 1874-1878, see abstract.	1-18, 28-36, 56-61

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 February 1992	26 MAR 19[illegible]

International Searching Authority	Signature of Authorized Officer
ISA/US	Scott A. Chambers

Form PCT/ISA/210 (second sheet) (Rev.11-87)